Bats and roads: Evaluating methods for risk assessment

Submitted by Amy Grace Fensome, to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, in July 2018

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

(Signature) ……

Abstract

In this thesis I present my investigation into the impact of roads on bats in the UK.

The road network is expanding rapidly around the world. Road infrastructure is expected to increase globally by an additional 25 million kilometres by 2050, an increase of 60% above 2010 levels.

In the UK, road density is already extremely high. There are 395,000 kilometres of road, 50,000 kilometres of which are major roads. Roads divide the UK into 325,369 patches so that almost half the UK's landcover is divided by roads into patches of less than 5km^2. The UK's road network is expected to expand by a further 640 kilometres of motorways and major roads by 2020.

Roads have a wide range of impacts. Roads are a direct cause of habitat loss and fragmentation. Roadside habitats can also be considerably altered by noise, light and chemical pollution, changes to light and temperature regimes and the hydrological cycle. Roads also pose a direct threat to biodiversity; a wide range of taxa are killed by vehicles on roads.

Bats may be vulnerable to the impact of roads. UK bat species use a much larger area for foraging than predicted for a mammal of their body size, typically within a radius of 1-6 km of the roost, depending on the species. The area within which bats forage is referred to as the core sustenance zone (CSZ), and for UK species the CSZ area can range from 3-112 km^2.

Due to their large range requirements, bats are likely to encounter roads frequently. If bats cross roads they are at risk of collision with vehicles. If bats do not cross roads, they may be limited to foraging within a suboptimal amount of space.

I used a three-step procedure, examining the behavioural, ecological and physiological responses of bats to roads to provide an integrated assessment of the threats posed by roads to bats.

First, I performed a systematic review of the literature and conducted meta-analyses to assess the threat posed by roads to bats as a result of (1) collisions with vehicles and (2) as a barrier to movement.

Second, I tested whether the distribution of bat roosts in the UK relates to the size of patches created by major roads. I also examined the influence of patch quality including the density of minor roads, and the area of woodlands, grassland and built environments. To determine whether road-defined patches were occupied by at least one roost I used the locations of 6199 bat roosts obtained from Natural England and the National Bat Monitoring Programme (NBMP).

Finally, I investigated whether corticosteroid levels, as exhibited by individuals within maternity roosts, correlated with a range of environmental variables including proximity to the nearest road and road density within the core sustenance zone.

At step one, I found that major roads are more likely to be a barrier to movement than minor roads and that they influence the distribution of bat roosts in the UK. At step two, it was determined that roosts were less likely to be located in smaller patches as defined by major roads and were more likely to be found in patches with a greater area of woodland. Step three reveals a possible correlation between the density of

minor roads within a roosts core sustenance zone and corticosteroid and gonadal steroids exhibited by members of that roost.

This three step procedure could be applied to other genera where studies have been published on road crossing behaviour (Step 1), where the presence/absence and location of focal animals and their nests have been extensively recorded, and from which it is relatively easy to obtain faecal samples, especially without causing additional stress or distress for the focal animals.

Table of Contents

List of Tables

excluded; ●=study reported this species, o= study took place within the geographical range of this species but no individuals were found; x= study took place outside this species' range.

Table 2.4 The number of bat carcasses by species reported in each study. In total, 1207 bats were recovered from roads in Europe. The one study from the USA (Russell et al., 2009) that reported casualties was excluded. The number of months during which surveys took place is shown in parentheses after the country, except for the following papers which collated incidental records from multiple sources over the following periods: 1945-1995 (Haensel & Rackow 1996); 1964-1993 (Kiefer et al. 1995); 1951-1993 (Rackow et al. 1994).

Table 2.5 The flight height category to which each reported species included in the meta-analysis was assigned.

Table 2.6 Site characteristics as reported by authors. This data was not available for three studies (Rackow et al. 1994, Kiefer et al. 1995, Haensel & Rackow, 1996) and so they have not been included.

Table 2.7 Articles retrieved during our literature search relating to roads as barriers to the movements of bats.

Table 2.8 Results reported by Bennett & Zurcher (2013) of the number of bats crossing in the presence and absence of vehicles and the height at which they were observed crossing.

Table 2.9 Results reported by Bennett & Zurcher (2013) of the number of bats crossing in the presence and absence of vehicles and the presence or absence of a tree layer.

Table 3.1 The expected and observed number of roosts (a) and proportion of occupied patches (b) for each aggregated quartile and the results of Pearson's chi-squared tests.

Table 3.2 The summed area of patches that contain zero, one or multiple roosts.

Table 3.3 The average area of road-defined patches containing zero, one or multiple roosts and the results of ANOVAs.

Table 3.4 The results of a Games Howell *post hoc* test confirmed a significant difference between the average size of patches containing zero and one roost, zero and multiple roosts and one and multiple roosts in the first aggregated quartile. Specifically, patches with one roost were larger than patches with zero roosts, and patches with multiple roosts and larger than patches with a single roost.

Table 3.5 The mean values of occupied and unoccupied patches for each aggregated quartile for minor road density (Figure 3.5 a) broadleaved woodland (Figure 3.5 b), coniferous woodland (Figure 3.5 c), grassland (Figure 3.5 d), and built environments (Figure 3.5 e).

Table 3.6 The top three models obtained by multi-model inference as well as the odds ratios and confidence intervals for each variable in the top model.

Table 4.1 The physiological and behavioural changes mediated by glucocorticoids during an acute short-term stress response (adaptive) and as a result of prolonged, heightened levels in response to a chronic stressor (maladaptive). Collated from Landys et al. 2006; Reeder & Kramer 2005; Sapolsky et al.2000; Sheriff et al. 2011.

Table 4.2 The effect of road related variables (total = 8) on baseline glucocorticoid levels reported in 18 studies (references in Appendix 4.2). Out of a total of 26 correlations between baseline glucocorticoid levels in wild living animals and road related variables or comparisons between individuals exposed to road related variables and controls, 13 were associated with an increase or higher glucocorticoids, 11 reported no effect and just two reported a negative correlation. Research effort refers to the total number of times each variable has been reported. A single study (4) reported the same effect for two different species; wolves (*Canis lupus*) and elk (*Cervus elaphus*).

Table 4.3 Twenty-six comparisons between male and female baseline and stress-induced glucocorticoid levels reported in eight species from eight studies. All individual comparisons are reported for cortisol and corticosterone. Where authors compared combined cortisol and corticosterone levels, I have referred to them as glucocorticoids. Pink squares denote that that females had higher glucocorticoid levels, blue denotes males had higher glucocorticoid levels and white squares denote no significant difference between sexes. The letter P denotes a comparison between males and

pregnant females, the letter L denotes a comparison between males and lactating females and the letter H denotes a comparison between males and females preparing for hibernation.

Table 4.4 The predicted direction of correlations between faecal corticosteroids and reproductive hormones (RH) for each of 17 predictor variables, including 9 directly related to roads, 4 indirectly related to roads (habitat quality) and 4 related to weather conditions. The basis for each prediction as a consequence of each variable's influence on the energy available in the environment (E_G), the energy invested in accessing resources (E_I), the minimum energy required to maintain homeostasis (E_E) or the potential for additional perturbances such as psychological stimuli from a perceived threat from vehicles or artificial lighting (E_O) are also noted.

Table 4.5 A list of target compounds including corticosteroids and reproductive hormones or their metabolites and precursors to both corticosteroids and reproductive hormones.

Table 4.6 The results of intercorrelations between target compounds. For each intercorrelation, the result of Pearson's correlation is reported on the left side of the column and the result of Spearman's correlation is reported on the right. Results highlighted in light pink squares are significant (<0.05) and those highlighted in dark pink are highly significant (<0.01).

Table 4.7 Rotated component loadings for each principle component. The percentage variance that each component accounts for is provided in brackets.

Table 4.8 The Eigen value of each principle component as well as the proportion of variance accounted for by each principle component and cumulative variance.

Table 4.9 Pearson's correlation coefficients between environmental variables and target compounds (a) and principle components (b). Significance is reported for both Pearson's (P) and Spearman's (S) correlations. Light pink indicates significance at the 0.05 level and dark pink indicates significance at the 0.01 level.

Table 4.10 The direction of the correlation between road density (all and minor) and each compound within PC 1 and PC 4; positive (+) and negative (-). The percentage variance that each component accounts for is shown in brackets.

Table 4.11 The treatment protocol used for this experiment.

Table 4.12 The number of samples for each treatment for each species and both species combined.

Table 4.13 The number of samples for each species and both species combined.

Table 4.14 The number of samples in each paired t-test. It was not possible to conduct t-tests for *P. auritu*s due to insufficient samples.

Table 4.15 The results of paired samples t-tests between each treatment category for *R. hipposideros* and for *R. hipposideros* and *P. auritus* combined.

Table 4.16 A list of target compounds including corticosteroids and reproductive hormones or their metabolites and precursors to both reproductive hormones and corticosteroids.

Table 4.17 The results of intercorrelations between target compounds. For each intercorrelation, the result of Pearson's correlation is reported on the left side of the column and the result of Spearman's correlation is reported on the right. Results highlighted in light pink squares are significant (<0.05) and those highlighted in dark pink are highly significant (<0.01).

Table 4.18 Rotated component loadings for each principle component. The percentage variance that each component accounts for is provided in brackets.

Table 4.19 The Eigen value of each principle component as well as the proportion of variance accounted for by each principle component and cumulative variance.

Table 4.20 The results of Mann-Whitney U tests on comparisons between *M. daubentonii* and *M. nattereri* for each target compound

List of Figures

Figure 3.6 The proportion of occupied patches in each bin for patch size (a), minor road density (b), broadleaved woodland area (c), coniferous woodland area (d), grassland areas (e) and the area of built environment (f).

Figure 3.7 The probability of a roost being present within a road-defined patch of a given size, with 1, 5, 50 and 100% broadleaved woodland within the patch. All other variables are set at their mean values (coniferous woodland 1.5%; grassland 20%; minor road density 0.003 m/m^2) except for built environments which was set at 20% coverage within a patch rather than a mean of 50% for the sake of clarity. The geographical co-ordinates for the hypothetical patches are for a location in the South West of England.

Figure 3.8 The change in the probability of roosts being present in two new equally sized road-defined patches following fragmentation of the orginal patch by a new road. I include estimates for patches with 10, 20 and 50% built environments. All other variables have been set at their mean (broadleaved woodland, 5%; coniferous woodland, 1.5%; grassland, 20%; minor road density, 0.003 m/m^2).

Figure 3.9 The change in the probability of roosts being present in two new equally sized road-defined patches following fragmentation of the orginal patch by a new road. I include estimates for patches with 5, 10 and 50% broadleaved woodland. All other variables have been set at their mean (built environments 50%; coniferous woodland 1.5%; grassland 20%; minor road density 0.003 m/m^2).

Figure 3.10 The number of potentially erroneous replicates (light grey) out of all roosts in each quartile (dark and light grey). The proportion of all roosts that these potential replicates represent are also provided.

Figure 3.11 The number of roosts in each quartile for the original analysis, including potentially erroneous replicates (a; n=6192), and without (b; n=5226). The total number of patches in each analysis, n=13893.

Figure 3.12 The number of roosts in each aggregated quartile in England (a; n=4633) and Scotland (b; n=273). Roads create 11,144 patches in England and each aggregated quartile is 32,692 ± 51 km^2. Roads create 1831 patches in Scotland and each aggregated quartile is 17,134 ± 261 km^2.

Figure 4.1 The HPA axis and the pathway of glucocorticoids at basal, modulated and stress induced levels.

Figure 4.2 Diagram demonstrating how glucocorticoids operate within three overlapping systems; basal, modulated and stress induced glucocorticoids (recreated from Busch & Hayward 2009).

Figure 4.3 A framework for modelling energetic requirements of organisms during the course of a year, where E_E represents the minimum amount of energy required to maintain homeostasis (*Equilibrium*); E_I is the energy *invested* in maintaining homeostasis in response to predictable changes; E_G is the energy *gained* in the environment that is available to the animal to eat; and E_O represents an additional event or disturbance that places an additional energetic demand (*ordeal*) on the organism such as increasing the energy required to obtain food. Figure 4.3 a provides a simplistic hypothetical scenario where E_G, E_I and E_E remain constant over time. E_O represents an event (i.e. stimuli) that causes Type 1 allostatic overload (i.e. the stress response and the emergency life history stage). Figures 4.3. b and c include more realistic changes to E_G, E_I and E_E. Note that a stressful event (E_O) is more likely to trigger allostatic overload in winter. In winter, however, allostatic overload can be achieved if E_G drops below E_I (blue star, c). Adapted from McEwen and Wingfield (2003).

Figure 4.4 Summary of Type 1 allostatic overload. The emergency life history stage (ELHS) is trigger when $E_O + E_I + E_E$ exceed E_G. This causes the organism to modify its behaviour in order to rectify or avoid the cause of E_O which causes glucocorticoids to return to baseline levels. Adapted from McEwen and Wingfield (2003).

Figure 4.5 Two contrasting hypothetical scenarios where challenging conditions that incur an energetic cost (E_O) cause an increase in glucocorticoids. In the first scenario (a), the challenging conditions do not cause $E_O + E_I + E_E$ to exceed E_G and so allostatic overload Type 1 is not achieved. The challenging conditions subside, and glucocorticoids return to baseline levels. In the second scenario (b), the challenging conditions also do not cause Type 1 allostatic overload. However, the challenging conditions do not subside, and so glucocorticoids remain high. This leads to allostatic overload Type 2. Adapted from McEwen and Wingfield (2003).

Figure 4.6 The number of studies investigating endocrinological responses to conservation relevant variables; 81 comparisons within 71 studies (see Appendix 4.1 for references).

Figure 4.7 Exploring the impact of conservation relevant variables on HPA activity in wild animals could elucidate one mechanism by which population declines occur. (Reproduced from Fensome AG 2013).

Figure 4.8 The number of studies that have explored the impact of road related variables on glucocorticoids in wild living species by taxonomic group; birds = 10, mammals = 4, reptiles = 2, amphibians = 2, total = 18).

Figure 4.9 Scree plot of Eigen values for each principle component.

Figure 4.10 Ordination plots of principle components for PC 1 against PC 2 (a) and PC 3 against PC 4 (b). P. auritus pre-treatment n=2, P. auritus post-treatment n=6, R. hipposideros pre-treatment n=4, R. hipposideros post-treatment n=10.

Figure 4.11 Scatter plots of principle components and environmental predictor variables; PC 1 and the density of all roads (a), PC 1 and the density of minor roads (b), PC 1 and the percentage of broadleaved woodland within the CSZ (c), PC 1 and the percentage of broadleaved woodland within road-defined patches (d), PC 1 and average temperature (e), PC 4 and the density of all roads (f) and PC 4 and the density of minor roads (g). Only those correlations that are significant are presented here, all correlations including those that were not significant are provided in Appendix 4.22-4.38. Closed red circles = *P. auritus* open circles = *R. hipposideros*.

Figure 4.12 Scatter plots of intercorrelations between environmental predictor variables including the density of all roads and minor roads (a), the percentage of broadleaved woodland within CSZs and road-defined patches (b), and the density of minor roads and the percentage of broadleaved woodland within CSZs (c).

Figure 4.13 The typical position of lights relative to the focal roost. Lights were positioned on tripods either side of a known flight path, about 20m from the roost.

Figure 4.14 Average principle component scores for *R. hipposideros* and *P. auritus* combined, for PC 1 (a), PC 2 (b), PC 3 (c), PC 4 (d). Post-treatment 1 & 2 samples have been combined.

Figure 4.15 Average principle component scores for *P. auritus*, for PC 1 (a), PC 2 (b), PC 3 (c), PC 4 (d). Post-treatment 1 & 2 samples have been combined. There was only one sample for post-treatment score 3 which meant that an average and standard error could not be calculated.

Figure 4.16 Average principle component scores for *R. hipposideros*, for PC 1 (a), PC 2 (b), PC 3 (c), PC 4 (d). Post-treatment 1 & 2 samples have been combined.

Figure 4.17 *P. auritus* and *R. hipposideros* scores for PC 1 plotted against PC 2 (a) and PC 3 against PC 4 (b).

Figure 4.18 Scree plot of Eigen values for principle components.

Figure 4.19 Male and female *M. daubentonii* and *M. nattereri* scores for PC 1 plotted against PC 2 (a) and PC 3 against PC 4 (b).

Figure 4.20 A comparison of *M. daubentonii* and *M. nattereri* average scores for PC2 (a) and PC 3 (b).

Figure 4.21 *M. daubentonii* and *M. nattereri* scores for PC 1 plotted against PC 2 (a) and PC 3 against PC 4 (b).

Figure 4.22 A comparison of the average scores of *M. daubentonii* males and females for PC 3.

Figure 4.23 Male and female *M. daubentonii* scores for PC 1 plotted against PC 2 (a) and PC 3 against PC 4 (b) and male and female *M. nattereri* scores for PC 1 plotted against PC 2 (c) and PC 3 against PC 4 (d).

Figure 4.24 Average faecal pellet weight for *M. daubentonii* and *M. nattereri*.

Figure 4.25 Average faecal pellet weights for female and male *M. daubentonii* and *M. nattereri*.

List of Accompanying Material

Appendix 1.1 Systematic review of the literature pertaining to bats and roads

A total of 400 studies were assessed for relevance in two databases (Google Scholar and Web of Science). Studies were selected if they investigated the impact of roads in relation to bat ecology and behaviour. *In Google Scholar*, the first two hundred titles retrieved with the search terms “roads AND bats OR Chiroptera” were checked for relevant studies. In *Web of Science*, the first two hundred titles retrieved with the search terms “roads AND bats OR Chiroptera” were checked for relevant studies.

Appendix 2.1 The behavioural traits of each study species associated with behavioural response to roads. Low-flying, clutter adapted species and species that are more sensitive to artificial lighting are more likely to avoid roads. Low-flying species= <10m, high-flying species >10m.

Appendix 3.1 Roosts obtained from the National Bat Monitoring Programme and Natural England datasets by species and for each country.

Appendix 3.2 The number of occupied and unoccupied patches by country

Appendix 3.3 The number of roosts per quartile for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e).

Appendix 3.4 The proportion of patches occupied by at least one roost in each aggregated quartile for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e).

Appendix 3.5 Minor road density (m/m^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e).

Appendix 3.6 The log of broadleaved woodland area (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e).

Appendix 3.7 The log of coniferous woodland area (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e).

Appendix 3.8 The log of grassland area (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e).

Appendix 3.9 The log of built environments (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e).

Appendix 3.10 The expected and observed number of roosts in each aggregated quartile. * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

Appendix 3.11 The expected and observed proportions of occupied patches in each aggregated quartile and the results of Pearson's chi-squared tests for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e). * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

Appendix 3.12 Comparisons of occupied and unoccupied patches in each aggregated quartile for minor road density broadleaved woodland, coniferous woodland, grassland and built environments. Results are reported for each species. * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001

Appendix 3.13 The number of roosts per quartile with 0-80% built environments (a), 0-20% built environments (b) and 80-100% built environments (c).

Appendix 3.14 The proportion of patches occupied by at least one roost in each aggregated quartile for patches with 0-80% built environments (a) and 0-20% built environments (b) and 80-100% (c).

Appendix 3.15 Minor road density (m/m^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for patches with 0-80% built environments (a), 0-20% built environments (b), and 80-100% built environments (c).

Appendix 3.16 The log of broadleaved woodland area (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for patches with 0-80% built environments (a), 0-20% built environments (b) and 80-100% built environments (c).

Appendix 3.17 The log of coniferous woodland area (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for patches with 0-80% built environments (a), 0-20% built environments (b) and 80-100% built environments (c).

Appendix 3.18 The log grassland area (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for patches with 0-80% built environments (a), 0-20% built environments (b) and 80-100% built environments (c).

Appendix 3.19 The log of built environments (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for patches with 0-80% built environments (a), 0-20% built environments (b) and 80-100% built environments (c).

Appendix 3.20 The expected and observed number of roosts for each aggregated quartile and the results of Pearson's chi-squared tests for patches with 0-80%, 0-20% and 80-100% built environments. * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

Appendix 3.21 The expected and observed proportions of occupied patches in each aggregated quartile and the results of Pearson's chi-squared tests for patches with 0-80% built environments (a) and 0-20% built environments (b). * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

Appendix 3.22 The mean values of minor road density, broadleaved woodland, coniferous woodland, grassland and built environments in occupied and unoccupied patches with 0-80%, 0-20% and 80-100% built environments. * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

Appendix 4.1 Systematic review of literature pertaining to endocrinological and other stress related responses to conservation relevant variables

Appendix 4.2 Systematic review of the literature pertaining to the impact of roads on glucocorticoids in wild living animals

Appendix 4.3 A complete list of target compounds including those not identified

Appendix 4.4 Calibration curves for aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j) and β-estradiol (k).

Appendix 4.5 The % relative standard error of samples.

Appendix 4.6 The bionsynthesis pathway of steroid hormones.

Appendix 4.7 Distance to nearest road correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.8 Distance to nearest A road correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.9 Distance to nearest B road correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.10 Distance to minor road correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.11 Density of all roads correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.12 Density of A roads correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.13 Density of B roads correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.14 Density of minor roads correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.15 CSZ broadleaved woodland (%) correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.16 CSZ built environment (%) correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.17 Road-defined patch size correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.18 Patch built environment (%) correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.19 Average temperature correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g),

pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.20 Average minimum temperature correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.21 Average wind speed correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.22 Average rainfall correlated against aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

Appendix 4.23 Distance to nearest road of any type correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.24 Distance to nearest A road correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d

Appendix 4.25 Distance to nearest B road correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.26 Distance to nearest minor road correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.27 Density of all types of road correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.28 Density of A roads correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.29 Density of B roads correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.30 Density of minor roads correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.31 CSZ broadleaved woodland (%) correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.32 CSZ built environment (%) correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.33 Road defined patch area correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.34 The area of broadleaved woodland within road defined patches, as a percentage, correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.35 The area of built environments within road defined patches as a percentage, correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.36 Average temperature correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.37 Average minimum temperature correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.38 Average wind speed correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.39 Average rainfall events correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

Appendix 4.40 Correlations between PC 1 and the density of all roads (a) and the percentage of broadleaved woodland within the CSZ (b) were moderately correlated but not significant (Pearson's correlation coefficient).

Appendix 4.41 The dates on which samples were collected. All samples were collected between June and September 2015 whilst bats roosted in maternity roosts.

Appendix 4.42 Calibration curves for aldosterone (a), androsterone (b), cortisone (c), corticosterone (d), dihydrotestosterone (e), hydrocortisone (f), pregnenalone (g),

progesterone (h), testosterone (i), 1-dehydrotestosterone (j), 11-deoxycorticosterone (k), 17α-hydroxyprogesterone (l)

Appendix 4.43 Comparisons between *M. daubentonii* and *M. nattereri* average concentrations of target compounds; aldosterone (a), androsterone (b), corticosterone (c), cortisone (d), dihydrosterone (e), hydrocortisone (f), pregnenolone (g), progesterone (h), testosterone (i), 1-dehydrotestosterone (j), 11-deoxycorticosterone (k), 17α-hydroxyprogesterone (l).

Appendix 4.44 Comparisons between *M. daubentonii* and *M. nattereri;* PC 1 (a), PC 2 (b), PC 3 (c), PC 4 (d).

Appendix 4.45 Comparisons between. *M. daubentonii* and *M. nattereri* males and females for each target compound; *M. daubentonii* aldosterone (a), androsterone (b), corticosterone (c), cortisone (d), dihydrotestosterone (e), hydrocortisone (f), Pregnenolone (g), progesterone (h), testosterone (i), 1-dehydrotestosterone (j), 11-deoxycorticosterone (k), 17α-hydroxyprogesterone (l); *M. nattereri*, aldosterone (m), androsterone (n), corticosterone (o), cortisone (p), dihydrotestosterone (q), hydrocortisone (r), Pregnenolone (s), progesterone (t), testosterone (u), 1-dehydrotestosterone (v), 11-deoxycorticosterone (w), 17α-hydroxyprogesterone (x).

Appendix 4.46 Comparisons between males and females for each principle component; *M. daubentonii* PC1 (a), PC 2 (b), PC 3 (c), PC 4 (d); *M. nattereri* PC1 (e), PC 2 (f), PC 3 (g), PC 4 (h).

Author's Declaration

The contribution to the chapters in this thesis by the main author, Amy G. Fensome (AGF) are shown below with contributions indicated from Danielle Linton (DL), Debbie Salmon (DS), Fiona Mathews (FM), James Cresswell (JC) and Vicky Fowler (VF). For additional assistance please see Acknowledgements.

Chapter 1

Original draft: AGF; Review and editing JC

Chapter 2

Conceptualization: AGF, FM; Statistics: AGF; Original draft: AGF; Review and editing: FM.

Chapter 3

Conceptualization: AGF; Data collection: AGF; Statistics: AGF, JC; Original draft: AGF; Review and editing: JC

Chapter 4

Conceptualization: AGF, FM; Data collection: AGF and DL collected faecal samples from *M. daubentonii* and *M. nattereri*; AGF and VF collected samples from *P. auritus* and *R.hipposideros;* AGF and DS extracted and quantified endocrinological targets; Statistics: AGF, JC; Original draft: AGF; Review and editing: JC

Chapter 1: General Introduction

Thesis pp 41 – 71

1.0 Chapter 1: General Introduction

1.1 Summary

In this chapter, I establish a basis for my research, which is to develop a three-step risk assessment procedure that could identify at-risk species as targets for conservation intervention. As an example, I consider the anthropogenic threat posed by roads, which is important because roads have a wide range of impacts. Roads are a direct cause of habitat loss and fragmentation. Roadside habitats can also be considerably altered by noise, light and chemical pollution, changes to light and temperature regimes and the hydrological cycle. Roads also pose a direct threat to biodiversity; a wide range of taxa are killed by vehicles on roads. Moreover, the road network is expanding rapidly around the world. Road infrastructure is expected to increase by an additional 25 million kilometres by 2050, an increase of 60% above 2010 levels. In particular, I focus on the effects of landscape fragmentation due to roads.

As a focal species, I use bats because they may be sensitive to landscape scale habitat alteration by roads. Roads may be one potential cause of declines or changes in distribution. The impacts of roads on bats may be complex and include direct and indirect impacts. Impacts are likely to be species dependent; i.e. some species may be vulnerable to roads as a barrier whilst other species may be more vulnerable to collisions with vehicles. Impacts are also likely to be dependent on road characteristics; major roads may be more likely to have a barrier effect than minor

roads; and on characteristics of the surrounding habitat such as the presence and proximity of roadside vegetation.

Traditionally the impacts of roads and other anthropogenic disturbances have focused on their influence on population abundance and distribution, or the immediate behavioural response of animals to the disturbance. However, an increasingly important technique to evaluate stress is to study the impact of anthropogenic disturbances on an organism's physiological response, such as the endocrinological state.

I studied glucocorticoids, which are hormones that mediate the stress response at high levels. They also rise and fall according to energetic demands and availability due to their role in promoting foraging behaviour. Roads could elicit heightened glucocorticoid levels by inducing a stress response as a result of stimuli such as moving vehicles, traffic noise or artificial lighting. Additionally, roads could be associated with heightened glucocorticoids by reducing the quality or availability of resources.

In this chapter, I review the evidence that chronically high glucocorticoids in an organism are associated with a range of deleterious physiological impacts which may have downstream impacts at the population level, which affect demographic sustainability. By measuring glucocorticoid levels in bats in relation to road related variables, I argue that it may be possible to identify the mechanisms by which roads cause declines.

In summary, there are two primary aims for this thesis. The first is to develop a three-step risk assessment procedure that could be applied to other species in order

to identify at-risk species. The second is to conduct a pilot study which will enable us to improve upon the current study.

1.2 Roads

1.2.1 Expansion of the road network

The road network is expanding rapidly around the world. Road infrastructure is expected to increase by an additional 25 million km by 2050, an increase of 60% above 2010 levels (Winn et al. 2011).

In Europe, over 630,000 km of new roads were built Between 2004 and 2013, approximately 70,000 km each year (European Commission 2013). The European Commission is currently overseeing the investment of €24 billion in the transport network by 2020 (European Commission 2014). By 2050, projections suggest there will be over 50 million km of paved road in Europe (Figure 1.1; Winn et al. 2011).

In the UK, for example, the road network is extremely dense and has grown rapidly since the 1960s (Figure 1.2). There are 395,000 km of road of which 50,000 km are major roads (Department for Transport 2017b). Overall, there is 1.7 km/km^2; England has the highest road density at 2.3 km/km^2 whilst Scotland has the lowest road density at 0.75 km/km^2 (Department for Transport 2017b). The UK government plans to invest more than £15 billion by 2020 on 127 road-building projects, including 640 additional kilometres of motorway and major road widening schemes (Department for Transport 2014).

1.2.2 Why it is important to study the impacts of roads in the UK

Remarkably, the UK is broken into 325,369 patches bordered by roads (data not shown). These patches, or 'road-bordered islands', vary widely in size: 89% are less than 1 km^2 which equates to 13% of the UKs landcover; 97% of patches are less than 5 km^2 accounting for 47% of the UKs landcover; and just 0.05% are greater than 100 km^2 or 30% of the UKs landcover. The important consequence of this widespread fragmentation is the large amount of habitat-road border, or edges, which make large areas susceptible to 'edge effects'. A review of 282 studies found that all of the impacts of roads described were reported to be experienced within 1 km of roads, whilst 14% occur within 5 km (Ibisch et al. 2017). This suggests that roads are a defining feature of the UKs landscape. At least 47% of the UKs landcover is within the impact zone of multiple roads.

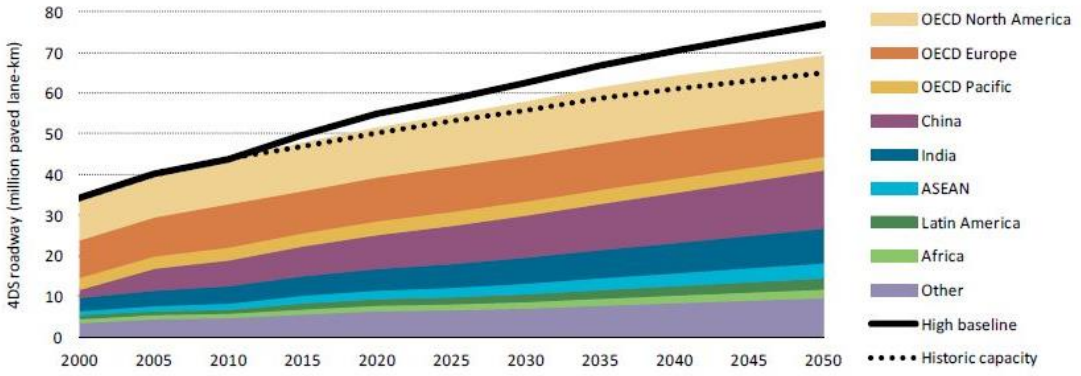


Figure 1.1 Projected increase in the kilometres of paved roads up to 2050 (Dulac 2013)

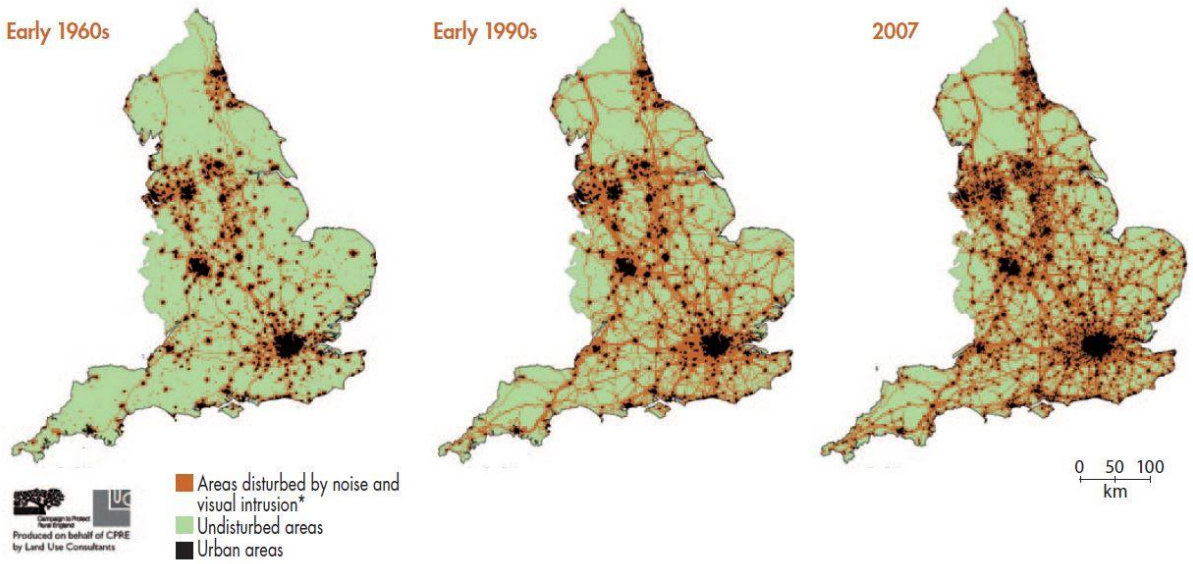


Figure 1.2 Areas of urban intrusion in the 1960s, 1990s and 2007 (Winn et al. 2011).

1.2.3 Ecological impacts of the road network

Roads have a wide range of impacts on the organisms that inhabit the landscapes that border them. For wild populations that live in landscapes permeated by road networks, abundances decline with proximity to roads in a wide range of taxa (Fahrig & Rytwinski 2009; Benítez-López et al. 2010; Karlson & Mörtberg 2015; Torres et al. 2016; Galantinho et al. 2017), which suggests that roads cause detrimental impacts, even to the extent that they can threaten biodiversity. Some cases may be explained by road-related mortality (Fahrig & Rytwinski 2009), which is extensive amongst amphibians (Fahrig et al. 1995; Glista et al. 2008; Hels & Buchwald, 2001), reptiles (Chittaragi & Hosetti, 2014; Heigl et al. 2017; Sosa & Schalk, 2016), birds (Erritzoe et al. 2003; Kociolek et al. 2011), invertebrates (Shyama Prasad Rao & Saptha Girish 2007) and mammals (Langbein 2011; Fensome & Mathews 2016).

89 Roads can also have indirect impacts on population abundance and
90 persistence by reducing the availability, accessibility and quality of habitats in their
91 vicinity (Trombulak & Frissell 2001; Coffin 2007). For example, roads not only occupy
92 previously habitable area, they also facilitate the encroachment of human
93 developments (e.g. so-called 'ribbon developments') leading to further loss and
94 degradation (Trombulak & Frissell 2001; Coffin 2007). Roadside habitats can be
95 considerably altered by chemical pollution from the emissions of vehicles that use
96 them, changes to light and temperature regimes, and disturbance by artificial lighting
97 (e.g. street lights and car head lamps) and traffic noise (Trombulak & Frissell 2001;
98 Coffin 2007) producing edge effects that can exert an influence on habitats up to 5 km
99 from the road (Benítez-López et al. 2010; Ibisch et al. 2017).

100 For some species, roads act as a partial or complete barrier to movement of
101 wild animals (Coffin, 2007; Trombulak & Frissell, 2001), thereby creating landscapes
102 that appear to be geographically continuous but are ecologically fragmented. For
103 example, many wild mammals are less likely to cross roads where traffic is continuous
104 or frequent. In consequence, roads can have the effect of fragmenting landscapes into
105 patches, which could have profound ecological impacts. For example, animal species
106 may become vulnerable meta-populations with local extinction dynamics or restricted
107 gene flow may also have deleterious effects. For species that avoid roads, the degree
108 of road fragmentation will determine patch sizes, which could influence the availability
109 of accessible resources (e.g. the areas of 'home ranges') and hence the viability of
110 resident populations (Jaeger et al. 2005; Eigenbrod et al. 2007). Restrictions on patch
111 size caused by roads will be particularly problematic for species with large home
112 ranges (Fahrig & Rytwinski 2009; Torres et al. 2016) and species dependent on
113 'interior habitats' (as patch size decreases, the area of core habitat relative to edge

habitat decreases; Bender et al. 1998). For example, the edges of roads can be dominated by light-dependent vegetation that is excluded from closed cover, which may be a less favourable habitat for 'interior' species. By acting as a barrier to movement, roads could prevent recolonization of depopulated fragments (Forman & Alexander 1998) or reduce gene flow between isolated populations (Holderegger & Di Giulio 2010).

1.3 Roads and bats

1.3.1 How the ecology of bats in the UK make them vulnerable to the impact of roads

1.3.1.1 Area requirements of bats

There are 18 species of bat in the UK (Table 1.2); 17 of which breed in the UK and one migrant species (Schofield & Mitchell-Jones 2011). Bats use a range of different habitat types for roosting and foraging. Some species roost in trees, caves and houses whilst foraging within or at the edge of woodland, over cattle grazed fields or over ponds (Schofield & Mitchell-Jones 2011). Their reliance on a range of different habitat types for roosting and foraging typically means that individuals must commute over large distances (Schofield & Mitchel-Jones 2011).

Compared to non-flying mammals, UK bat species use a much larger area for foraging than predicted for a mammal of their body size (Kelt & Vuren 1999), typically foraging within a radius of 1-6 km of the roost, depending on the species (Bat

Conservation Trust UK 2016). The area within which bats typically forage is referred to as the 'core sustenance zone' (CSZ), and for UK species the CSZ area can range from 3-112 km^2 (Table 1.2; Bat Conservation Trust UK, 2016).

Bats in the UK use different roosts in summer (e.g. maternity roosts) and winter (i.e. hibernation roosts), which can be considerable distances apart (Schofield & Mitchel-Jones 2011). Within a season, bats may switch between several roosts (Schofield & Mitchel-Jones 2011). Consequently, the wide range of travel undertaken by many bats will expose them to encountered with road edges that are so prevalent across the UK's landscape.

1.3.1.2 Feeding habits

All species of bat in the UK are insectivorous (Schofield & Mitchel-Jones 2011). Insects are an ephemeral food source; i.e. the location and abundance of insect aggregations is variable. This may be one reason why bats use such large areas for foraging. Many species forage for insects within the interior or at the edges of woodland, especially broadleaved woodland (Schofield & Mitchel-Jones 2011). The area of woodland in the UK has declined drastically (Schofield & Mitchel-Jones 2011) and what remains is highly fragmented – and in part by roads.

1.3.1.3 How spatial and feeding habits make bats vulnerable to the impact of roads

Given that bats switch between roosts within and between seasons, the sizeable area within which bats forage and the density of roads in the UK, it is likely

155 that bats encounter roads frequently. Many species of bat are reliant on woodland,
156 which has decreased in total area, is increasingly fragmented and is likely to be
157 bisected by roads, which suggests that bats may be vulnerable to the impact of roads:
158 either bats must cross roads in order to access sufficient resources and in so doing
159 risk collision with vehicles; or they avoid roads and are limited to the resources
160 confined within a patch bordered by roads. Whether and to what extent roads pose a
161 threat to bats will depend in part on their response to roads which is likely to be
162 species-specific and may be dependent on various road characteristics such as width,
163 traffic density, or the presence of artificial lighting.

164 **1.3.1.4 Known impacts of roads on bats**

165 Relatively little research has focused on the impact of roads on bats. My review
166 of 36 studies reveals that to date much of the research focuses on relatively simple
167 questions such as whether bats are killed on roads and what factors influence the
168 behavioural response of bats to roads (i.e. approach or avoid).

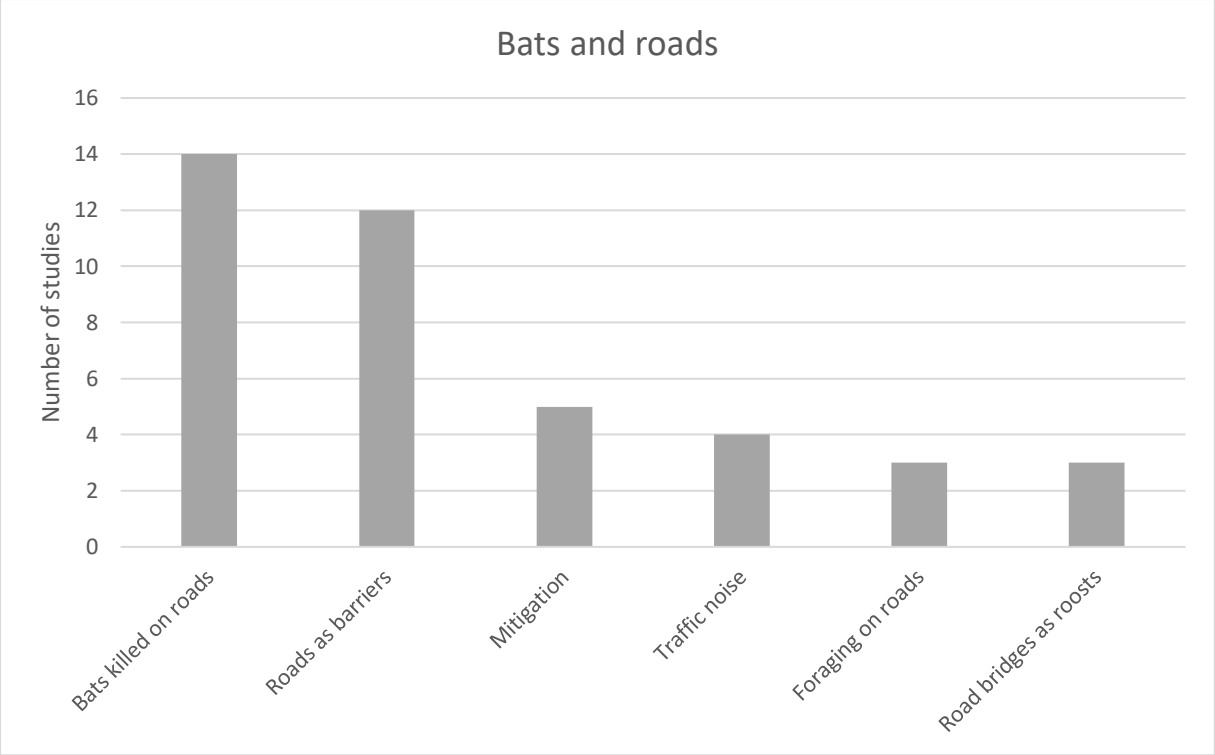


Figure 1.3 The number of studies that have investigated the impact of roads on bats or reported behaviours such as foraging on roads or roosting in road bridges (n=36 studies, references in Appendix 1.1).

1.3.1.5 Collisions with vehicles

Bats that cross roads are at risk of collisions with vehicles. Fourteen studies have reported multiple incidences of bats killed on roads (Figure 1.3; Appendix 1.1). Most studies examine or report factors relating to the spatial distribution of road-killed bats including characteristics of roads and the surrounding environment, such as road width, the presence of traffic and the presence and proximity of road side vegetation. In Chapter 2, I conduct a review and meta-analysis of studies and reports published before 2015 (i.e. prior to the date when the chapter was published) in order to identify

key risk factors including how species traits and an individual's sex influence collision risk.

1.3.1.6 Roads as barriers

Bats that avoid roads are at risk of being cut off from the wider landscape. As a barrier to landscape scale movement, roads could prevent bats from accessing sufficient resources (e.g. food and roosts) and isolate breeding populations, both of which could potentially cause population declines. Twelve studies to date have explored the potential for roads to act as a barrier to landscape scale movement (Figure 1.3; Appendix 1.1). These studies examine the influence of characteristics of roads and surrounding habitat on whether bats cross or avoid roads. In Chapter 2, I review how the behavioural response of bats to roads (i.e. cross or avoid) is influenced by road related characteristics (e.g. the presence of vehicles, traffic noise, light pollution and road width), the surrounding habitat (e.g. the presence and proximity of bordering vegetation) and species traits (e.g. the height at which a species typically flies or foraging strategy).

1.3.1.7 Roads as a useful habitat; foraging and roosting over roads

It is not clear that roads are invariably negative in their effects on bats because several species of bat have been observed foraging on roads, which may indicate a preference for roadside habitats. Most reports are from unlit roads (Myczko et al. 2017) but two species have been observed foraging around street lamps on roads (Blake et al. 1994; Rydell 1992). The density of traffic on these roads was probably low; the

198 authors report that surveyed roads were residential or rural (Blake et al. 1994; Rydell
199 1992) or that traffic was low to moderate (Myczsko et al. 2017) but do not provide
200 exact numbers of passing vehicles during the survey period.

201 Roads may be useful foraging spaces for insectivorous bats. Roads, especially
202 minor roads in rural areas, are often bordered by hedgerows and trees and are
203 therefore structurally identical to the linear features along which many of species of
204 bat are adapted to forage (Altringham 2011; Down & Racey 2006). Roads might also
205 increase prey availability and predictability; the dark surface of roads absorbs heat
206 during the day and slowly releases heat during the night which attracts insects
207 (Myczko et al. 2017 and references therein). Residential roads are often bordered by
208 artificial lights which also attract insects (Rydell 1992), although these aggregations
209 can only be exploited by bat species that are not sensitive to artificial lighting (Mathews
210 et al. 2015). Myczko et al. (2017) propose that passing traffic could disorientate insects
211 due to sudden changes in air pressure, temperature and lighting, whilst traffic noise
212 could mask the sound of approaching bats, making it easier for bats to catch insects.

213 The infrastructure associated with roads can also provide roosting habitats.
214 *Nyctalus noctula*, *Myotis myotis, M. daubentonii* and *Pipistellus pipistrellus* are
215 amongst the European species that have been reported to roost in road bridges
216 (Cal'Uch and Ševčik 2008, and references therein). Bridge roosts have also been
217 reported in North America (Bennett et al. 2009; Allen et al. 2010). A comparison
218 between Brazilian free-tailed bats (*Tadarida brasiliensis*) roosting in caves and road
219 bridges revealed that bridge-dwellers were less stressed and had better body
220 condition than bats living in caves (Allen et al. 2010). The authors suggest that bats
221 living in bridge roosts may be less sensitive to noise and artificial lighting, and that they

may have lower stress levels because they have a lower ectoparasite load due to roosting at lower densities under bridges. They also observed that bridge dwelling bats spent less time away from the roost possibly because they are closer to food sources and so expend less energy foraging and thermoregulating.

Clearly, the interactions between bats and roads can be complex and difficult to interpret. For example, it is possible to argue that the very attractiveness of roads to some bats, (i.e. when roads can be useful habitats for foraging and roosting) make roads an ‘ecological trap’. Specifically, the trap works because bats are attracted to foraging along roads and around street lights, and roosting under bridges, but they may as a consequence be more susceptible to collisions with vehicles

Factor	Impact		Individual	Roost	Species	Species traits
	(+)	(-)				
Collisions with vehicles		Unsustainable additional mortality could cause population declines	Review (Ch. 2)		Review (Ch. 2)	Review (Ch. 2)
Distance of roost to nearest road		Bats roosting closer to a road may experience more disturbance and greater risk of collision		Endocrinology (Ch. 4)		
Roadside lighting	Some bats forage on aggregates of insects around light	Some species avoid artificial lighting, limits movement and foraging space		Endocrinology (Ch. 4)		
Roadside vegetation	Used for commuting, foraging also aids road crossing	Could increase risk of collision with vehicles			Review (Ch. 2)	Review (Ch. 2)
Road surface	Warmed surface attracts aggregates of insects	Could increase risk of collision with vehicles				
Road width	Wider roads could be less permeable, reducing available foraging space, and isolating populations	Wider roads might be avoided by bats, reduces collision risk.			Review (Ch. 2)	Review (Ch. 2)
Road bridges	Useful roosting space, aid road crossing and so reduce negative impacts of barrier effects					
Road density		The greater the density of roads the greater the level of disturbance by roads and traffic (e.g. light, noise) and the greater the likelihood of habitat fragmentation or collisions with roads.		Road-defined patches (Ch. 3) & endocrinology (Ch. 4)	Road-defined patches (Ch. 3)	

Traffic noise	Traffic noise could encourage bats to avoid roads and so reduce likelihood of collisions with vehicles	Traffic noise could encourage bats to avoid roads and so increase the barrier effect			Review (Ch. 2)	Review (Ch. 2)
Traffic density	A greater density of traffic could encourage bats to avoid roads and so reduce likelihood of collisions with vehicles	Greater traffic density could encourage bats to avoid roads and so increase the barrier effect, or bats that do not avoid roads may be more likely to be killed by vehicles			Review (Ch. 2)	Review (Ch. 2)

Table 1.1 A summary of factors related to the interactions of bats with roads, the potential negative and positive effects of this factor and the scale at which this factor is studied and the methodology and chapter in which it is studied.

1.3.1.8 Traffic noise

Noise from passing vehicles may deter some species of bat from flying close to roads, crossing roads or foraging in the vicinity of roads, which reduces available habitat. Bats are less likely to cross the road in the presence of passing vehicles, especially if the vehicle produces noise above 88 dB (Zurcher et al. 2010). In *Myotis myotis* (the greater mouse-eared bat), traffic noise reduces both foraging activity (Schaub et al. 2008) and foraging efficiency (Siemers and Schaub 2010). For example, traffic noise could impair the ability of *Myotis myotis* to forage by masking the sound of insect prey (Schaub et al. 2008; Siemers and Schaub 2010). The 'masking effect' could also explain the differential impact of traffic noise on species with different foraging strategies; slow-flying, gleaning species (species that rely on passive listening to detect prey) tend to forage further away from traffic than fast flying species that actively use echolocation to detect their prey (Bonsen et al. 2015). Consequently, roads may have differential impacts among bat species.

1.3.1.9 Infrastructure aimed at mitigation of road impacts: gantries and underpasses

Gantries are often included in the 'mitigation design' of new roads. These structures span the road and, where possible, link linear traditional commuting features used by bats on either side of the road. However, recent work shows that gantries are probably largely ineffective as the few bats that have been observed crossing at gantries do not increase the height at which they cross the road (Berthinussen & Altringham 2012a).

Under-road passageways are another means of potentially enabling bats to cross roads safely. Their efficacy may depend on their width and is possibly limited to species with certain traits. Specifically, ‘clutter’ and ‘edge adapted’ species, may be best served by under-road passages, than ‘open adapted’ species, especially if those passages are relatively wide.

‘Clutter adapted’ species (i.e. those that typically forage within woodland) have slower and manoeuvrable flight adaptive to foraging in the interior of woodlands (after Abbott et al 2012b; Bhardwaj et al. 2017; Ceľuch et al. 2008 and references therein). They also tend to forage and commute close to the ground which may explain why they preferentially use under road routes. ‘Edge adapted’ species forage on the edges of woodland and in and around trees (after Abbott et al 2012b; Bhardwaj et al. 2017; Ceľuch et al. 2008 and references therein). They are faster fliers and their flight height is more variable which could explain why they will use wider under-road routes. ‘Open adapted’ species fly faster and higher compared to the other two species guilds and seem to consistently prefer over road routes (after Abbott et al 2012b; Bhardwaj et al. 2017; Ceľuch et al. 2008 and references therein).

Clutter adapted species were more likely to use wide road bridges and narrow culverts than to fly directly over the road whereas edge adapted species are more likely to cross under a wide road bridge than to fly over the road, but preferred to fly over the road than to use a narrow culvert (Ceľuch et al. 2008). Open adapted species were more likely to fly over the road than to use either road bridges or culverts (Bhardwaj et al. 2017). Similarly, clutter-adapted species such as *Rhinolophus hipposideros*, *Myotis nattereri* and *Plecotus auritus* were the only species observed flying through narrow under road pipes whilst edge-adapted species (e.g. species from

the *Pipistellus* genus) were very active in the area but appeared to avoid flying through the pipes. The same study demonstrated that all species recorded in the area used a wider under road passageway, with the exception of *Nyctalus leisleri*, an open adapted, high-flying species (Abbott et al 2012b).

1.3.1.10 Population changes in bats and the need for conservation

Of the 17 species that breed in the UK, the abundance of five species is increasing, five species are in decline, one species appears to be stable whilst the status of the remaining six is unknown (Table 1.2). It is possible that roads have directly and indirectly contributed to these declines as those species for which a decline has been reported appear to be more vulnerable to the impacts of roads (Table 1.2). Declining species are more likely to be woodland specialists (two declining species versus one increasing species) which could make them more vulnerable to habitat loss and fragmentation by roads. Declining species are more likely to forage on unlit roads (five declining species versus three increasing species) which could increase the risk of collisions with cars. Additionally, declines are most severe in bat species that are most vulnerable to fragmentation caused by roads. Specifically, two of the five declining species have CSZs with an area of 50 km^2, whereas all of the increasing species use a CSZ of 28 km^2 or less. Species with greater habitat requirements could encounter roads more frequently and therefore be more susceptible to collisions with vehicles, or they may be more sensitive to habitat limitation as a result of the barrier effect of roads. In Chapter 2, I demonstrate that low-flying clutter adapted species are more likely to avoid roads. Three of the declining

300 species are low-flying clutter adapted species, compared to two of the increasing
301 species.

Species	Light sensitivity	Woodland specialists	Forage on roads	Exhibit characteristics associated with road avoidance	CSZ radius (km) and area (km^2)	Population size (1995)	Population trend (2010-2016)
Pipistrellus pipistrellus	Forage		●x		2 km, 12 km^2	2,000,000	Up (FW)
Pipistrellus pygmaeus	Forage		x		3 km, 28 km^2	NA	Up (FW)
Myotis brandti	Avoid		x	x	1 km, 3 km^2	30,000	Up (H)
*Rhinolophus hipposideros**	Avoid	X		x	2 km, 12 km^2	14,000	Up (H)
Rhinolophus ferrumequinum	Avoid				3 km, 28 km^2	4000-6600	Up (H)
Myotis nattereri	Avoid	X	x	x	4 km, 50 km^2	100,000	NC (H)
*Plecotus auritus**	Avoid	X	x	x	3 km, 28 km^2	200,000	Down (H)
Myotis daubentonii	Avoid		x	x	2 km, 12 km^2	150,000	Down (FW)
Myotis mystacinus	Avoid		x	x	1 km, 3 km^2	40,000	Decline (JNCC)

Nyctalus noctule	Forage	X	●x		4 km, 50 km^2	50,000	Down (FW)
Eptesicus serotinus	Forage		x		4 km, 50 km^2	15,000	Down (FW)
Nyctalus leisleri	Forage				3 km, 28 km^2	10,000	Unknown
Barbastella barbastellus	Forage	X	x	x	6 km, 112 km^2	5000	Unknown
Pipistrellus nathusii	Forage		x		3 km, 28 km^2	Unknown	Unknown
Myotis bechsteinii	Avoid	X		x		1500	Unknown
Myotis alcathoe	Avoid			x	1 km, 3 km^2	Unknown	Unknown
Plecotus austriacus	Avoid		x	x	3 km, 28 km^2	Unknown	Unknown

Table 1.2 Summary of key ecological and behavioural traits of bat species in the UK that could indicate vulnerability to the impacts of roads, including sensitivity to artificial lighting (Reviewed in Day, 2017); woodland habitat specialism (Bat Conservation Trust 2017); observations of foraging on roads (Blake et al. 1994; Rydell 1992; Myczko et al. 2017); whether the species shares attributes associated with road avoidance behaviour (i.e. low-flying, clutter-adapted species are more likely to avoid roads, reviewed in Chapter 2); core sustenance zone size (Bat Conservation Trust, 2016); population size estimates (Harris et al. 1995) and population trends

(Bat Conservation Trust, 2017; except for *Myotis mystacinus (Harris et al. 1995*)). Where two population size estimates were reported (Harris et al. 1995) the upper estimate is shown. Estimates were not available for some species in 1995. For example, *Myotis alcathoe* was first identified in the UK in 2010. *Pipistrellus pygmeus* was not identified as a separate species from *Pipistrellus pipistrellus* until 1997 so the population estimate for *Pipistrellus pipistrellus* probably includes both species. For species that have been reported to forage on roads, those that were reported foraging over artificially lit roads are indicated by circles (Blake et al. 1994; Rydell 1992) and unlit roads are indicated by crosses (Myczko et al. 2017).

1.4 Thesis aims

The need to examine the impact of roads is pressing. Globally the road network is expanding rapidly and is expected to increase to 60% above 2010 levels. In the UK, road density is already extremely high, and further development is expected in the near future. A large area of landcover in the UK is within the zone of influence of roads. To ensure that appropriate and effective mitigation is incorporated during the planned period of intensive road expansion, or to prevent further expansion if necessary, it is essential to understand how roads effect bats and other animals.

1.4.1 Why bats are a useful focal group

Bats are a useful focal species for studying the impacts of roads because bats use large habitat ranges and are likely to encounter roads frequently. Preliminary research suggests that roads pose a double threat to bats: they have the potential to act as a barrier whilst bats that do cross roads are vulnerable to collisions with vehicles. Whether and in what way a road poses a threat depends on several interacting factors including characteristics of the road, surrounding habitat and species traits. Bats appear to respond behaviourally to the presence of roads, and they are either threatened directly by traffic or the quality and abundance of their forage may suffer. Overall, therefore, bats are potentially a sentinel species for any widely-ranging animal whose habitats are fragmented by roads.

1.4.2 Linking the behavioural responses of bats to roads to the distribution of bat roosts via a physiological marker

With regard to the impact of roads on wild animals, obvious and common initial areas of research include investigating the response of animals to roads (i.e. cross or avoid); reporting whether they are killed on roads and examining what factors influence collision risk; and modelling the influence of roads on the distribution or abundance of populations. In other words, it is logical to examine the immediate indicators of a potential disturbance at the level of individuals (i.e. the behavioural response, mortality) in order to inform and test hypothesise about the downstream effects of a disturbance at the population level (i.e. abundance or distribution).

In this thesis I examine upstream indicators (i.e. the behavioural response of bats to roads) and downstream effects (the distribution of roosts in relation to roads). A major goal is to attempt to identify a potential mechanistic link between the two by examining the physiological response of bats to roads. Specifically, I measure the endocrinological response of bats to roads.

Assessing the endocrinological state of free-living animals is an emerging and increasingly important method of investigating the impact of anthropogenic disturbance (Ellis et al. 2012; Keay et al. 2006; Sheriff et al. 2011; Wikelski & Cooke 2006 and references therein). One group of hormones that are often the focus of such studies are the glucocorticoids and their metabolites (Sheriff et al. 2011). At heightened levels, glucocorticoids mediate the stress response. At moderate levels they play a role in regulating energy, rising and falling daily and seasonally in response to changes in energetic demands such as arousal from sleep, foraging, breeding and hibernation (Landys et al. 2006; McEwen & Wingfied 2003; Sheriff et al. 2011).

345 Glucocorticoids are potentially an ecologically valuable indicator because
346 anthropogenic disturbance that reduces an organism's ability to meet its energetic
347 demands or that causes an emergency stress response, cause glucocorticoid levels
348 to rise (Ellis et al. 2011; McEwen & Wingfield 2003; Bradshaw 2007). Roads could
349 elicit heightened glucocorticoid levels as a result of stimuli such as traffic noise or
350 artificial lighting and/or by limiting access to resources (see Table 4.2 and references
351 therein; Ellis et al. 2011). The first reason to examine stress hormones is, simply, that
352 they present a useful indicator of an individual animal's health, which may in itself have
353 a demographic interpretation. Additionally, the stress hormones may have a directly
354 causal role in demographic outcomes. For example, chronically elevated
355 glucocorticoids can have negative impacts on the physiology and behaviour of
356 organisms including reproduction, the immune system and tissue growth and healing
357 (see Table 4.1). Hence, roads could cause population declines if stimuli associated
358 with roads, or reduced food availability, cause glucocorticoids to be chronically
359 elevated.

360 Thus, the endocrinological response of bats to roads could be the underlying
361 mechanism (and indicator) that links observations with regards to their behavioural
362 response to roads (i.e. avoidance) to the population level effect (i.e. preferential
363 roosting in larger patches).

364 Here, I evaluate the possibility that stress hormones in general, and
365 glucocorticoids in particular, could be used to evaluate the level of stress caused by
366 an anthropogenic stressor such as fragmentation due to the presence of roads. If bats
367 avoid roads it suggests that they perceive certain road attributes as noxious stimuli,
368 and glucocorticoids could increase as part of the stress response (Sheriff et al. 2011).

These stimuli also contribute to the barrier effect of roads by triggering road avoidance. The barrier effect could reduce the availability of food, which is also associated with an increase in glucocorticoids (Busch & Hayward 2009), though not necessarily at levels associated with the emergency stress response.

Therefore, the downstream effect observed at the population level could be that bats are more likely to roost in larger patches. This could occur via two non-mutually exclusive pathways; bats are more likely to roost in larger patches because roosts in smaller patches do not persist due to the harmful effects of heightened glucocorticoids on survival and reproduction. Alternatively, bats actively select larger road defined patches in order to reduce exposure to road related stimuli and to maximise the available foraging space without having to cross roads.

Investigating the potential impact of roads on the behaviour, distribution and physiology of bats not only provides an integrated picture of how roads could potentially impact bats, but could also constitute a repeatable three-step risk assessment that could be used to identify and target other at-risk species or taxonomic groups.

1.5 Thesis aim: To design a three-step procedure for studying the impacts of roads on animals.

1.5.1 Step 1: Review evidence related to the potential for roads to act as a barrier to landscape scale movement.

Most existing research on the impact of roads on bats has focused on vehicle collisions and roads as barriers. I review this evidence to explore which characteristics of roads, surrounding habitat and species traits are associated with road crossing behaviour (and collision risk) or avoidance of roads, which is important in deciding whether, in actuality, roads fragment the landscape for bats.

As an example, my study will demonstrate that major roads are more likely to discourage road crossing behaviour than minor roads. Major roads are more likely to act as a barrier to movement as they tend to be wider, with more traffic, less bordering vegetation and a greater likelihood of being artificially lit.

My work serves as an exemplar of Step 1 because it ought to be relatively easy to replicate this step for other terrestrial animals. Whether and under what conditions animals cross or avoid roads, and whether they are struck by vehicles is an obvious first step when investigating the impact of roads on biodiversity. As such it seems likely that for many taxa, there will be a sufficiently large body of literature on behavioural responses to roads to determine whether roads are a partial or complete barrier to a taxonomic group or perhaps a specific species. Indeed, a quick search of Scopus (21st December 2018) with the search terms detailed in Table 1.3, retrieved 19 relevant studies for a review of roads as a barrier to bats. A similar or greater number of studies

were identified for other potential focal species or groups; 15, 38 and 16 studies appeared to be relevant from the title or abstract, to a review on roads as a barrier to badgers, deer and red fox, respectively (Table 1.3). Studies were deemed potentially relevant if the study addressed roads as a potential barrier to movement by the focal species or group. Additional searches in other publication databases such as Web of Science, EBSCO, CAB Abstracts or Google Scholar, as well as checking the bibliographies of relevant articles, are likely to expand the pool of relevant studies. These are, therefore, minimum estimates of the available literature to review.

These rapid reviews revealed that, just as with bats, roads can be a barrier to movement for deer (D'Amico et al. 2015), badgers (Clarke et al. 1998) and possibly foxes (Kato et al. 2016), and that roads pose a significant threat to all groups as a result of collisions with vehicles (Clarke et al. 1998; Grilo et al. 2015; Jakubas et al. 2018). Authors investigated several of the same key factors related to barrier effects and collision risk as addressed in similar studies of bats. For example, habitat quality influences collision risk for all groups (Jakubas et al. 2018; Meisingset et al. 2013). Traffic density influence both the barrier effect and collision risk; collisions peak at moderate traffic densities for red foxes (Grilo et al. 2015) but are only marginally related to traffic density for deer (Kämmerle et al. 2017; Kušta et al. 2017). Traffic density enhances the barrier effect for badgers (Clarke et al. 1998) and foxes (Grilo et al. 2015). It seems likely, therefore, that the literature is available to conduct similar reviews and analyses as presented in Chapter 2.

Focal species/group	Search terms	Number of retrieved studies in Scopus*	Potentially relevant
Bats	((road* OR highway* OR motorway* OR vehicle*) AND (bat OR bats OR Chiroptera) AND (barrier OR permeability OR cross OR crossing))	55	19
European badger	((road* OR highway* OR motorway* OR vehicle*) AND (badger* OR “Meles meles”) AND (barrier OR permeability OR cross OR crossing))	24	15
Native and non-native deer species found in UK	((road* OR highway* OR motorway* OR vehicle*) AND ("Fallow deer” OR “Red deer” OR “Roe deer” OR “Sika deer” OR “Muntjac” OR "Dama dama" OR “Cervus elaphus” OR “Capreolus capreolus” OR “Cervus nippon” OR Muntiacus) AND (barrier OR permeability OR cross OR crossing))	51	38
Red fox	((road* OR highway* OR motorway* OR vehicle*) AND (“red fox” OR “Vulpes vulpes”) AND (barrier OR permeability OR cross OR crossing))	19	16

Table 1.3 The number of retrieved and potentially relevant studies identified in Scopus for four example systematic searches. Studies were deemed potentially relevant if the title or abstract indicated that the study addressed the barrier effects of roads in relation to the focal species. (* searches conducted on 21/12/2018).

1.5.2 Step 2: If major roads are a barrier, is there a patch size effect on distribution?

By acting as a barrier to movement, major roads could determine the availability of accessible resources. This being so, and given the large habitat requirements of bats, it is possible that bats are more likely to roost in larger patches as defined by major roads. Below, I test whether the distribution of bat roosts in the UK is related to the size of patches created by major roads and examine the influence of patch quality including the density of minor roads, and the area of woodlands, grassland and built environments.

To determine whether road-defined patches were occupied by at least one roost I used the locations of 6199 bat roosts obtained from Natural England and the National Bat Monitoring Programme (NBMP) and used ArcGIS (ESRI v 10.3 2014) to map their location and calculate the size and quality of road-defined patches.

The results of my study begin to validate the feasibility and value of a 'cartographic ecology' of the road network. This finding is important because it will be possible to replicate this step for other taxonomic groups or species. For example, it may be possible for UK species to use datasets from the Biological Records Centre (BRC). The BRC collate species observation records from regional Wildlife Trusts.

1.5.3 Step 3. Do steroid levels in bats respond to road-related variables?

The first aim of Step 3 was to develop and test faecal glucocorticoid analysis as a tool for examining the impact of roads on bats.

Answers to the following questions will guide future studies, enabling us to expand and improve upon the current study.

1. Which hormones are present in faecal samples?
2. Are there differences between species, sexes and how much variation is there between individuals?
3. Can target compounds be correlated against environmental variables or do levels change following experimental treatment?

The second aim was to test experimentally whether bats roosting in smaller patches and/or in landscapes with a high density of roads, had lower gonadal steroid levels and/or higher corticosteroid levels thereby potentially reflecting the availability of resources and the extent to which bats are exposed to stress-inducing stimuli such as moving vehicles, traffic noise and artificial lighting. My findings begin to establish the value of faecal endocrinology as a method for evaluating the conservation status of bats.

Chapter 2: Roads and bats: a meta-analysis and review of the evidence on vehicle collisions and barrier effects

Thesis pp 74 – 118

2.0 Roads and bats: a meta-analysis and review of the evidence on vehicle collisions and barrier effects

This chapter was published as:

Fensome AG and Mathews F (2016) Roads and bats: a meta-analysis and review of the evidence on vehicles collisions and barrier effects. *Mammal Review,* 46, 311-323.

It has been cited 14 times as of May 2018.

2.1 Summary

Roads are a potential threat to bat conservation. In addition to the direct risk of collision with vehicles, they may prevent movement through the landscape. We performed a systematic review of the literature and conducted meta-analyses to assess the threat posed by roads to bats as a result of (1) collisions with vehicles and (2) as a barrier to movement. We collated records of 1,207 road casualties in Europe. We found that low flying species are more prone to collisions than high flying species, and also showed that juveniles are more vulnerable to collisions than adults. In addition, the meta-analysis identified a significant bias towards male casualties. This could be indicative of sexual segregation across habitats of varying quality, i.e. females may occupy better quality habitats where road density tends to be lower. It is also notable that casualties were not restricted to common species, but also included rare and declining ones such as *Barbastella barbastellus* and *Rhinolophus* species.

It is likely that the conditions that determine whether a road acts as a barrier are complex. For example, tree-lined roads with little traffic may offer good foraging habitat for some species. The presence of favourable habitat for bats (notably woodland) was a significant factor associated with a weaker barrier effect but a heightened risk of collision.

Our data suggest that there is substantial evidence that roads pose a threat to bats, especially as a result of collision. It is essential that the future expansion of the road network across Europe incorporates suitable and effective mitigation in order to minimise this threat. We conclude with suggestions for future avenues of research including the contribution of traffic noise and street lighting to the barrier effect of roads, and recommend that more consistent before-after studies should be conducted for new road schemes.

2.2 Introduction

The road network is expanding rapidly throughout Europe. Between 2004 and 2013, over 630000 km of new roads were built, an average of at least 70000 km each year (European Commission 2013). The network will expand further during the next five years as the European Commission oversees the investment of €24 billion in the transport network by 2020 (European Commission 2014), while the UK government plans to invest more than £15 billion on 127 road-building projects, including 400 additional miles of motorway and major road widening schemes (European Commission 2014).

503 There are several life history traits which place bats in general at high risk from
504 roads. Requiring a larger home range than would be predicted for a mammal of their
505 body mass (Kelt & Vuren 1999), they may be particularly sensitive to the loss and
506 fragmentation of habitat. Their use of different locations for foraging, mating,
507 hibernating and breeding, and their movements between summer roosts and
508 hibernation roosts (Schofield & Mitchell-Jones 2011) is also likely to elevate bat
509 encounter rates with roads. Bats are very long lived, and have low fecundity (1-2
510 offspring per year; Kunz & Fenton 2003), so may be unable to withstand even
511 moderate increases in mortality (Schorcht et al. 2009). In addition, many live at low
512 densities and have a patchy distribution, making them vulnerable to local extinction.

513 We examine the available evidence that roads pose a threat to bat populations
514 as a result of collisions between bats and vehicles, and by obstructing movement
515 across roads. How bats interact with roads (i.e. the extent to which they exhibit
516 avoidance behaviour) determines whether and how a road poses a threat. These
517 interactions are likely to be influenced by a number of factors including the behaviour
518 and ecology of bats, characteristics of the road, such as width and traffic volume and
519 the habitat characteristics in the vicinity of the road.

520 Within species, there may also be differences in collision risk. We predict
521 elevated risks in juveniles due to their inexperience of orientation, and in encountering
522 vehicles and artificial lights. Juveniles may also have slower and less manoeuvrable
523 flight as bats with high manoeuvrability tend to have low wing loadings, and it has been
524 shown in *Myotis lucifugus* that wing loading declines as juvenile bats grow (Adams
525 1996). It is also possible that juvenile bats prefer to forage or practice flying in the open
526 areas provided by roads. Habitat use has been shown to vary with age. For example,

527 juvenile *Myotis lucifugus* are more likely to forage in less cluttered habitats than adults
528 (Adams 1997).

529 There may also be sex differences in collision susceptibility, and the expected
530 effects may vary according to the time of year. Females could have a higher risk of
531 collision during late spring and early summer, when pregnancy and lactation means
532 they are heavier and less manoeuvrable, need to forage earlier and for longer, and
533 make regular returns to the roost to feed young. Conversely, in swarming species,
534 males are likely to be more susceptible to collision than females in the autumn,
535 because of the strong bias towards males visiting swarming sites, especially earlier in
536 the season (Parsons et al. 2003). Assuming a 1:1 sex ratio within a population as a
537 whole, male bats must visit more swarming sites than females and must make more
538 journeys in order to access these sites.

539 Many bat species use linear features such as hedgerows and tree-lines to orient
540 themselves between roosts and foraging sites (Altringham 2011) or for foraging
541 (Downs & Racey 2006). We predict that the presence of hedges or trees running
542 parallel with or perpendicular to a road could enhance the road's permeability to bats,
543 but also increase the likelihood of collisions between bats and vehicles, by
544 encouraging bat commuting or foraging activity.

545 Road width is likely to be negatively correlated with permeability for a number
546 of species: low-flying bats that tend to forage or commute within closed environments
547 (e.g. woodland) may be less likely to cross wide roads than species adapted to flying
548 high or to using open landscapes. Roadside lighting is similarly expected to have a
549 species-specific effect. Light is thought to deter slow-flying species (Stone et al. 2009)
550 whereas other species, such as *Pipistrellus pipistrellus,* use lit roads provided that tree

551 cover is available (Blake et al. 1994, Mathews et al. 2015). The risk of collision for
552 such species depends on the height of the lights and the height at which individuals
553 commute to and from the site.

554 The extent to which traffic might repel bats from the vicinity of roads is less
555 clear, but is likely to vary between species, according to their responsiveness to light
556 and noise, and may depend on traffic volume. Bats are thought to avoid locations with
557 loud background noise, as it interferes with their ability to use acoustic information to
558 locate insects, thereby reducing foraging efficiency (Schaub et al. 2008, Siemers &
559 Schaub 2011), and it may also affect commuting behaviour. Moving traffic may also
560 be perceived as a threat and induce predator avoidance behaviours (Baxter et al.
561 2006, Zurcher et al. 2010). These factors are not mutually exclusive and could deter
562 bats from the vicinity of roads. Alternatively, high traffic volumes could simply increase
563 the risk of collisions between bats and vehicles.

564 We systematically review the literature and, where data allow, conduct meta-
565 analyses to assess the threat posed to bats by roads through collisions and as barriers
566 to movement. We examine the composition of collated records of road-killed bats to
567 determine whether flight height, sex or age of individuals influences collision risk, and
568 we present data on the temporal and spatial distribution of mortalities. We assess the
569 evidence that road permeability depends on a species' foraging ecology, and is
570 influenced by characteristics of the road, such as width and traffic volume, as well as
571 by the ecology and topology in the vicinity of the road.

2.3 Evidence base

A systematic search was made of the Web of Knowledge, EBSCO and Google Scholar in February and March 2015 using the search terms “(road*OR highway* OR motorway* OR vehicle*)” and “(bat OR bats OR Chiroptera)” with either “(fatal* OR mortal* OR collision*OR casualty*)” or “(barrier OR permeability OR cross OR crossing)”. The bibliographies of relevant studies retrieved from this search were also checked for additional relevant studies.

Our study inclusion criteria were broad. Studies were selected for inclusion if they addressed either road crossing behaviour in bats or reported road casualties of bats. No other criteria determined inclusion or exclusion except for geographical location. Due to the paucity of data from elsewhere, and similarities in the composition and ecology of bat species within Europe and between Europe and the USA, the review was restricted to Europe and the USA. However, only two studies were conducted in the USA (Russell et al. 2009, Kitzes & Merenlender 2014). Russell et al. (2009) provide data which were included in the meta-analyses of sex and age. Sensitivity analyses confirmed that exclusion of this study from analyses had a minimal effect.

Through these searches we identified a total of 12 articles relating to bats as casualties on roads, and eight relating to the barrier effect of roads (Table 2.1). As the systematic review was thorough and inclusion broad, we are confident that the selection of data reviewed and analysed below are representative, if not complete. Conclusions drawn from this review, therefore, are unlikely to have been biased through the selection and inclusion process.

From the studies relating to collisions, we extracted data on the species, sex and age of road-killed bats, as well as the approximate date on which they were found. We also noted methodological information such as the length of the road surveyed and the duration of the study. The number of studies that took place within each species' range was recorded.

To facilitate comparisons between studies, casualty numbers are expressed as rates per kilometre of road per month. We were not able to calculate these figures for Germany, since data were derived from incidental records rather than systematic surveys of known stretches of road (Rackow et al. 1994, Kiefer et al. 1995, Haensel & Rackow, 1996).

Where data were sufficient, meta-analyses were conducted. We were able to conduct three separate analyses to examine potential differences in collision risk based on sex, age and flight height. Analysis of variation in collision risk over time was not possible since the date of carcass collection was rarely reported.

To permit assessment of the links between flight height and road impacts, we assigned species to either high-flying or low-flying categories (the category to which each species was assigned is shown in Table 2.6). High-flying species were defined as those usually flying more than 10 m above the ground (above the height of cars); low-flying species were defined as those that typically fly up to 5 m from the ground (Russ 1999). Forty-five individuals were not assigned to either category as they were not identified to species. Species in the genus *Pipistrellus* have variable flight heights but typically fly below 10 m (Russ 1999). They are also the most abundant and widespread group. For these reasons we decided to conduct the analysis both with

618 and without individuals from this genus; where they were included, we placed them in
619 the low-flying category.

620 Assuming that all adults in a population reproduce, and that most European
621 bats produce singleton offspring, the expected ratio of adults to juveniles is 2:1, thus
622 the expected proportion of juveniles within the population is 0.33 (this is a conservative
623 estimate; in many populations the proportion will be lower than 0.33 due to some adults
624 not breeding). The sex ratio was assumed to be 1:1 in all bat populations. The
625 expected ratio of low-flying to high-flying species could not be determined due to lack
626 of data, so it was not possible to test whether the observed ratio differed significantly
627 from that predicted by their abundance in the environment.

628 Meta-analyses were conducted in R version 3.1.2 (R Core Team 2014) using
629 the package “meta” (Schwarzer 2015). The analyses were based on binomial data
630 and therefore we used logit transformations and confidence intervals based on Wilson
631 Scores. The sensitivity of the analyses to the exclusion of individual studies was
632 tested.

633 In cases where insufficient data were available, or where there was large
634 variability in the definition of exposures (for example in assessments of habitats and
635 crossing points associated with casualty risks or barrier effects), a qualitative report of
636 the literature is provided instead of meta-analysis.

2.4 Results

We collated 1207 records of bat road casualties spanning five decades (Table 2.1). The country with the highest recorded number of casualties was Germany, where 464 individual bats were collated from records made over a 50-year period. The lowest number, among countries from which data were available, came from Montenegro, where 17 bats were recovered from roads during 14 months. Monthly casualties ranged from 0.03 bats km^{-1} in Montenegro (Iković et al. 2014) to 2.5 bats km^{-1} in the Czech Republic (Gaisler et al. 2009; Table 2.2). Most studies took place within the ranges of the species most frequently recorded as casualties (Table 2.3). Methodological variations account for some of the observed differences between studies, but site characteristics were also influential (see Table 2.1 and Table 2.6).

It is evident that casualties are not evenly distributed temporally or spatially. Researchers consistently reported mortality peaks during the mating and swarming season of each species (Rackow et al. 1994, Kiefer et al. 1995, Haensel & Rackow 1996, Lesiński 2007, Gaisler et al. 2009, Lesiński et al. 2010, Medinas et al. 2012) or during migration to winter roosts (Medinas et al. 2012; Figure 2.1).

Country	Method	Results	Reference
France	Roadside hedgerows searched weekly, on foot, May-October 1998-2002 (24 months)	Total 109	Capo et al. (2006)
Czech Republic	Emergency stopping lanes searched approx. weekly, on foot, May-October 2007 (6 months)	Total 119	Gaisler et al. (2009)
Spain	Lanes, hard shoulders and ditches searched weekly on foot, 1989 (12 months)	Total 72	Gonzalez-Prieto et al. (1993)
Germany	Collated incidental records, 1945-1995.	Total 307, 211 males and 96 females	Haensel & Rackow (1996)
Montenegro	Two roads searched weekly by bike, August-October 2013 (3 months)	Total 17, 8 males and 5 females	Iković et al. (2014)
Germany	Collated incidental records, 1964-1993	Total 96	Kiefer et al. (1995)
Poland	Approx. weekly searches, May-October 1994-2000 (36 months)	Total 167, 30 males and 31 females, 56 juveniles and 29 adults	Lesiński (2007) Method 1
Poland	Irregular searches on several roads, 1992-1993 and 2001-2004		Lesiński (2007) Method 2
Poland	Road side, searched approx. weekly, August-September 2004 and April-October 2005-2006 (16 months)	Total 44, 9 males and 15 females	Lesiński (2008)
Poland	Roadside, verges and ditches searched weekly, by car and by foot, July 2008-June 2009 (11 months)	Total 61, 20 males and 7 females, 17 adults and 8 juveniles	Lesiński et al. (2010)
Portugal	Daily search of several roads by car, March-October 2009 (7.5 months)	Total 154, 44 males and 20 females, 99 adults and 17 juveniles	Medinas et al. (2012)

Germany	Collated incidental records, 1951-1993	Total 61	Rackow et al. (1994)
Pennsylvania, USA	Road and verges, searched approx. weekly, on foot May-September 2001 (4 months)	Total 29, 4 males and 16 females, 12 adults and 15 juveniles	Russell et al. (2009)

Table 2.1 Articles retrieved during our literature search relating to collisions between bats and vehicles. Where the information has been provided by the authors, we note the frequency and location of searches, whether these were conducted on foot, by car or by bike, and the dates between which surveys took place. We include the total number of carcasses reported by each author as well as the proportions of both sex and age classes of individuals where these data were available.

Country	Total casualties	Combined length of roads surveyed (km)	Total survey time (months)	Casualties / km	Casualties / km/month
Czech Republic (1)	119	8	6	14.8	2.4
France (1)	104	2	24	52	2.1
Germany (3)	464				
Montenegro (1)	17	30	14.5	0.5	0.03
Poland (3)	225	25.6		8.7	1.4
Portugal (1)	154	51	3	3.	1
Spain (1)	72	17	12	4.2	0.3
USA (1)	29	0.1	3	193.3	64.4

Table 2.2 The total number of casualties reported by each country. The number in brackets refers to the total number of studies conducted in that country.

Species	**Capo et al. (2006)**	**Gaisler et al. (2009)**	**Gonzalez-Prieto et al. (1993)**	**Haensel and Rackow (1996)**	**Iković et al. (2014)**	**Kiefer et al. (1995)**	**Lesiński (2007)**	**Lesiński (2008)**	**Lesiński et al. (2010)**	**Medinas et al. (2012)**	**Rackow et al. (1994)**	**Number of studies within species' range**	**Number of studies where sp. found within range**
	France	Czech Republic	Spain	Ger-many	Mont-enegro	Ger-many	Poland	Poland	Poland	Portugal	Ger-many		
Rhinolophus blasii	X	X	X	X	●	X	X	X	X	X	X	1/11	1/1
Rhinolophus ferrumequinum	●	o	o	●	o	o	o	o	o	●	o	11/11	3/11
Rhinolophus hipposideros	o	o	●	●	●	●	●	o	o	●	●	11/11	7/11
Eptesicus nilsonii	o	o	X	●	X	●	o	o	o	X	o	8/11	2/8
Eptesicus serotinus	o	●	o	●	o	●	●	o	●	●	●	11/11	7/11
Nyctalus leisleri	o	●	o	●	o	●	●	o	●	●	●	11/11	7/11

Nyctalus noctule	o	●	o	●	o	●	●	o	●	o	●	11/11	6/11
Pipistrellus kuhlii	●	X	o	o	●	o	o	o	o	●	o	10/11	3/10
Pipistrellus nathusii	o	●	o	●	●	o	●	o	●	o	o	11/11	5/11
Pipistrellus pipistrellus	●	●	●	●	o	o	o	o	o	●	●	11/11	6/11
Pipistrellus pygmaeus	o	●	o	o	●	o	o	o	o	●	o	11/11	2/11
Pipistrellus savii	o	X	●	o	o	o	X	X	X	o	o	7/11	1/7
Myotis alcathoe	o	●	o	o	X	o	o	o	o	X	o	9/11	1/9
Myotis bechsteinii	●	o	o	●	o	●	o	o	o	o	o	11/11	3/11

Myotis brandtii	o	●	X	●	o	●	●	o	o	X	●	10/11	5/9
Myotis capaccinii	o	X	o	X	●	X	X	X	X	X	X	3/11	1/3
Myotis dasycneme	o	o	X	o	X	o	●	o	o	X	o	8/11	1/8
Myotis daubentonii	●	●	●	o	o	●	●	o	o	●	●	11/11	7/11
Myotis emarginatus	●	●	o	o	o	o	o	o	o	o	o	11/11	2/11
Myotis escalerai	o	X	o	X	X	X	X	X	X	●	X	3/11	1/3
Myotis nattereri	●	●	o	●	o	●	●	●	●	o	●	11/11	8/11
Myotis myotis	●	o	o	●	o	●	●	o	o	o	●	11/11	5/11

Myotis mysticanus	●	o	o	●	●	●	●	o	o	o	o	11/11	5/11
Barbastella barbastellus	●	o	o	●	o	●	●	o	●	●	o	11/11	6/11
Plecotus auritus	●	o	o	●	o	o	●	●	●	o	●	11/11	6/11
Plecotus austriacus	●	o	●	●	o	o	●	o	o	o	●	11/11	5/11
Vespertilio murinus	o	o	X	●	o	o	o	o	o	X	●	9/11	2/9
Miniopterus schreibersii	o	X	o	●	o	●	o	o	o	●	o	10/11	3/10

Table 2.3 The extent to which the combined studies are representative of each species of bat found in Europe. As such, studies conducted in the USA have been excluded; ●=study reported this species, o= study took place within the geographical range of this species but no individuals were found; x= study took place outside this species' range.

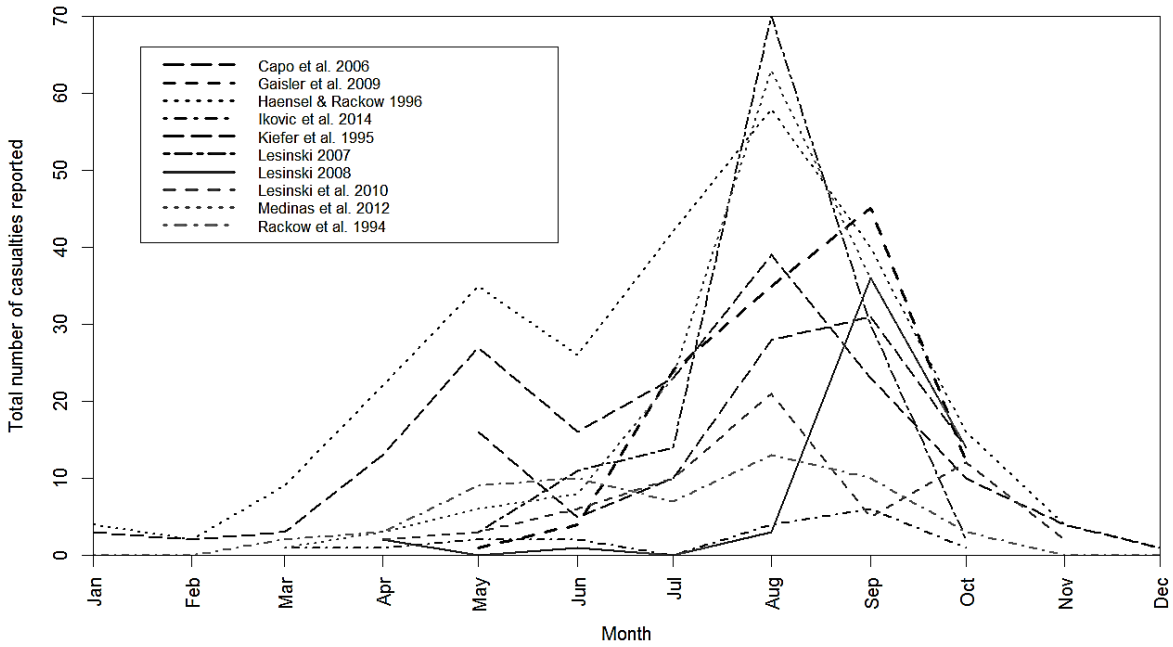


Figure 2.1 Line chart showing the numbers of casualties reported by each study at different times of the year. Ten studies reported the season in which carcasses were retrieved.

2.4.1 Collisions

2.4.1.1 Risk factors associated with the behaviour and ecology of bats

Of the 1207 recorded casualties, most were from the genera *Pipistrellus* (35%, n=419, from nine species) and *Myotis* (29%, n=351, from fifteen species; Table 2.4). Rarer species such as *Barbastella barbastellus* (2.3%, n=28), which is undergoing declines in parts of its range, were also recorded (IUCN, 2015).

Individuals from high-flying species (Table 2.5) represented 17% of all casualties (n=206). These included species from the *Nyctalus*, *Eptesicus* and

661 *Vespertilio* genera. In two studies, authors were able to relate the number of casualties
662 with local abundance estimates. They reported that *Nyctalus noctula* (Gaisler et al.
663 2009) and *Eptesicus serotinus* (Lesiński et al. 2010) formed a lower proportion of
664 casualties than would be predicted from their relative abundance estimated by
665 acoustic surveys (Gaisler et al. 2009) and netting (Lesiński 2007).

Species	**Capo et al. (2006)**	**Gaisler et al. (2009)**	**Gonzalez-Prieto et al. (1993)**	**Haensel and Rackow (1996)**	**Iković et al. (2014)**	**Kiefer et al. (1995)**	**Lesiński (2007)**	**Lesiński (2008)**	**Lesiński et al. (2010)**	**Medinas et al. (2012)**	**Rackow et al. (1994)**	**Total**
	France (24)	Czech Republic (5)	Spain (12)	Germ-any	Montene gro (14.5)	Germ-any	Poland (36)	Poland (16)	Poland (11)	Portugal (7.5)	Germ-any	
Rhinolophus blasii					1							1
Rhinolophus ferrumequinum	2			1						1		4
Rhinolophus hipposideros			14	3	4	2	2			7	1	33
Eptesicus nilsonii				3		2						5
Eptesicus serotinus		4		35		20	15		5	4	15	98
Nytalus leisleri		1		5		1	1		1	1	1	11
Nyctalus noctula		1		39		21	3		18		6	88

Pipistrellus kuhlii	4				6					67		77
Pipistrellus nathusii		32		2	1		3		3			41
Pipistrellus pipistrellus	47	2	33	83						21	12	198
Pipistrellus pipistrellus/ pygmaeus		8										8
Pipistrellus pygmaeus		32			3					45		80
Pipistrellus savii			2									2
Pipistrellus spp.	1	12										13
Barbastella barbastellus	6			4		3	2		10	3		28
Plecotus auritus	2			25			38	7	14		2	88
Plecotus austriacus	3		5	11			1				1	21
Plecotus spp.	4			4								8

Vespertilio murinus				3						1	4
Myotis alcathoe		1									1
Myotis bechsteinii	1			5		4					10
Myotis brandtii		1		1		1	6			1	10
Myotis capaccinii					1						1
Myotis dasycneme							2				2
Myotis daubentonii	14	19	18	17		9	62		3	5	147
Myotis emarginatus	7	2									9
Mytois escalerai									1		1
Myotis myotis	2			22		13	1			1	39
Myotis mysticanus	8			14	1	10	9				42

Myotis nattereri	2	1	7	6	12	37	6		2	73
Myotis spp.			3		8					11
Miniopteris schreibersii			1	1				1		3
Not determined	6	2	19		2		4		12	45

Table 2.4 The number of bat carcasses by species reported in each study. In total, 1207 bats were recovered from roads in Europe. The one study from the USA (Russell et al., 2009) that reported casualties was excluded. The number of months during which surveys took place is shown in parentheses after the country, except for the following papers which collated incidental records from multiple sources over the following periods: 1945-1995 (Haensel & Rackow 1996); 1964-1993 (Kiefer et al. 1995); 1951-1993 (Rackow et al. 1994).

Taxon	Flight height
Rhinolophus blasii	Low
Rhinolophus ferrumequinum	Low
Rhinolophus hipposideros	Low
Eptesicus nilsonii	High
Eptesicus serotinus	High
Nyctalus leisleri	High
Nyctalus noctula	High
Pipistrellus kuhlii	Low
Pipistrellus nathusii	Low
Pipistrellus pipistrellus	Low
Pipistrellus pygmaeus	Low
Pipistrellus savii	Low
Pipistrellus spp.	Low
Barbastella barbastellus	Low
Plecotus auritus	Low
Plecotus austriacus	Low
Plecotus spp.	Low
Vespertilio murinus	High
Myotis alcathoe	Low
Myotis bechsteinii	Low
Myotis brandtii	Low
Myotis capaccinii	Low
Myotis dasycneme	Low
Myotis daubentonii	Low
Myotis emarginatus	Low
Myotis escalerai	Low
Myotis lucifugus	Low
Myotis myotis	Low
Myotis mysticanus	Low
Myotis nattereri	Low
Myotis sodalis	Low
Miniopteris schreibersii	Low
Myotis spp.	Low

Table 2.5 The flight height category to which each reported species included in the meta-analysis was assigned.

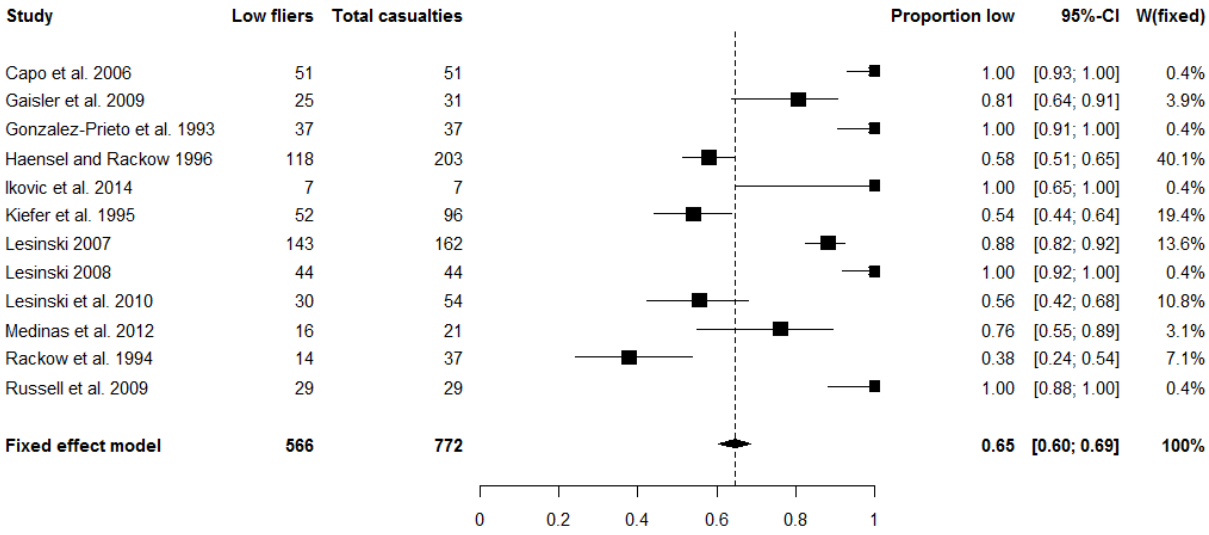
Meta-analysis demonstrated that most road casualties were attributed to low-flying species (excluding *Pipistrellus* individuals n= 566, including *Pipistrellus* individuals n= 985; Figure 2.2 a and b respectively). Sensitivity analysis showed that the exclusion of individual studies had little effect on this bias towards low-flying species (Figure 2.3 a and b). Of the 12 studies we identified during our systematic review, all but one documented a majority of low-flying species, while a further five studies did not document any high-flying species at all (Gonzalez-Prieto et al. 1993, Capo et al. 2006, Lesiński 2008, Russell et al. 2009, Iković et al. 2014).

Meta-analysis confirmed a highly significant overall bias towards males (332 males: 190 females; pooled confidence interval 0.59-0.68; Figure 2.2 c). The trend towards female casualties (16/20) observed in Russell et al.'s (2009) study is likely to be due to the proximity of the surveyed road to a known maternity roost. However, sensitivity analysis showed that the exclusion of individual studies had no effect on the overall bias towards males (see Figure 2.3 c). Of the 12 studies we identified during our review, in eight, the sex of casualties was reported. In four, a significant bias towards males was reported (Haensel & Rackow 1996, Lesiński et al. 2010, Medinas et al. 2012, Iković et al. 2014); in one, there was a non-significant trend towards males (Rackow et al. 1994). In one study, a significant bias towards females is reported (Russell et al. 2009) while in another, there is a non-significant trend towards females (Lesiński 2008), and in one study, no significant deviation from an equal sex ratio is reported (Lesiński 2007).

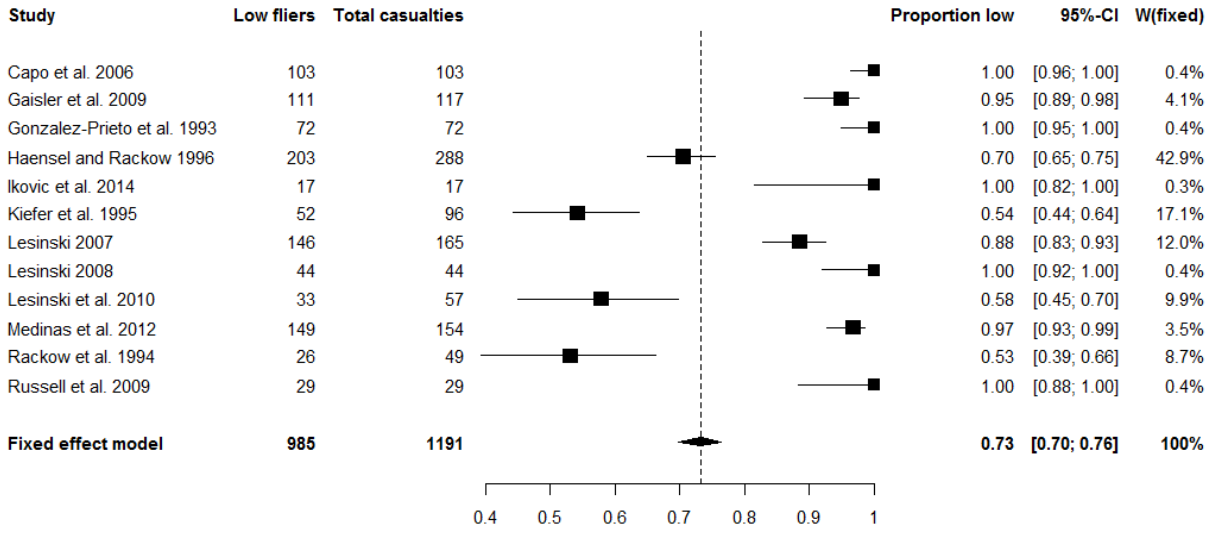
Meta-analysis showed that the observed proportion of juveniles was significantly higher than expected (pooled confidence interval 0.35-0.42), supporting our hypothesis that juvenile bats are more prone to collisions with vehicles than adults

690 (Figure 2.2 d). Sensitivity analysis demonstrated that the bias towards juveniles was
691 robust to the exclusion of individual studies; the mean proportion ranged from 0.34-
692 0.54, indicating that the proportion of juveniles is always greater or equal to their
693 expected abundance in the population (Figure 2.3 d). In five studies, information was
694 included on the age of bat carcasses found on roads. Of these, in two, a significant
695 bias towards juveniles was reported (Lesiński 2007, Russell et al. 2009), in another, a
696 non-significant bias towards juveniles was reported (Iković et al. 2014), while Lesiński
697 et al. (2010) report no significant deviation from the expected proportion, and in one
698 study a significant bias towards adults is reported (Medinas et al. 2012).

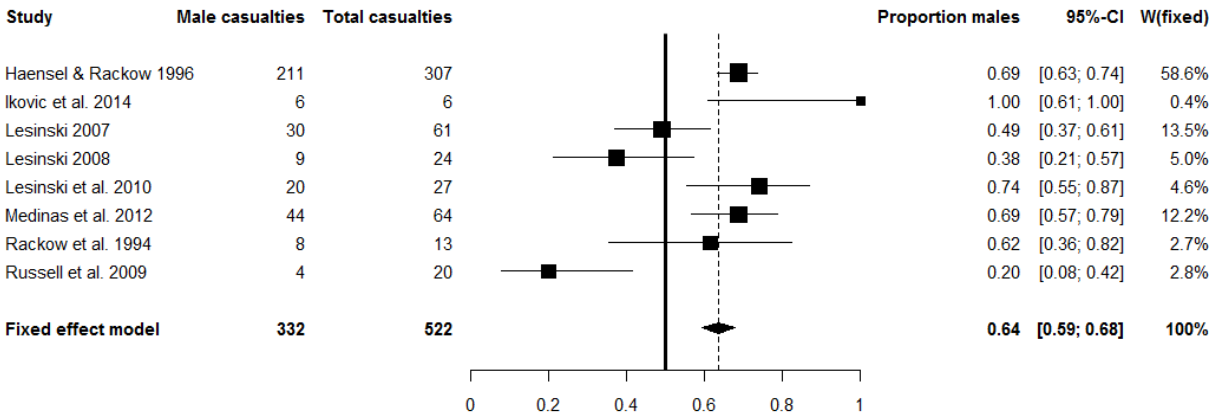
(a)


Study
Low fliers
Total casualties
Proportion low
95%-CI
W(fixed)
Capo et al. 2006 51 51 1.00 [0.93; 1.00] 0.4%
Gaisler et al. 2009 25 31 0.81 [0.64; 0.91] 3.9%
Gonzalez-Prieto et al. 1993 37 37 1.00 [0.91; 1.00] 0.4%
Haensel and Rackow 1996 118 203 0.58 [0.51; 0.65] 40.1%
Ikovic et al. 2014 7 7 1.00 [0.65; 1.00] 0.4%
Kiefer et al. 1995 52 96 0.54 [0.44; 0.64] 19.4%
Lesinski 2007 143 162 0.88 [0.82; 0.92] 13.6%
Lesinski 2008 44 44 1.00 [0.92; 1.00] 0.4%
Lesinski et al. 2010 30 54 0.56 [0.42; 0.68] 10.8%
Medinas et al. 2012 16 21 0.76 [0.55; 0.89] 3.1%
Rackow et al. 1994 14 37 0.38 [0.24; 0.54] 7.1%
Russell et al. 2009 29 29 1.00 [0.88; 1.00] 0.4%
Fixed effect model 566 772 0.65 [0.60; 0.69] 100%
0
0.2
0.4
0.6
0.8
1

(b)


Study
Low fliers
Total casualties
Proportion low
95%-CI
W(fixed)
Capo et al. 2006 103 103 1.00 [0.96; 1.00] 0.4%
Gaisler et al. 2009 111 117 0.95 [0.89; 0.98] 4.1%
Gonzalez-Prieto et al. 1993 72 72 1.00 [0.95; 1.00] 0.4%
Haensel and Rackow 1996 203 288 0.70 [0.65; 0.75] 42.9%
Ikovic et al. 2014 17 17 1.00 [0.82; 1.00] 0.3%
Kiefer et al. 1995 52 96 0.54 [0.44; 0.64] 17.1%
Lesinski 2007 146 165 0.88 [0.83; 0.93] 12.0%
Lesinski 2008 44 44 1.00 [0.92; 1.00] 0.4%
Lesinski et al. 2010 33 57 0.58 [0.45; 0.70] 9.9%
Medinas et al. 2012 149 154 0.97 [0.93; 0.99] 3.5%
Rackow et al. 1994 26 49 0.53 [0.39; 0.66] 8.7%
Russell et al. 2009 29 29 1.00 [0.88; 1.00] 0.4%
Fixed effect model 985 1191 0.73 [0.70; 0.76] 100%
0.4
0.5
0.6
0.7
0.8
0.9
1

(c)



(d)

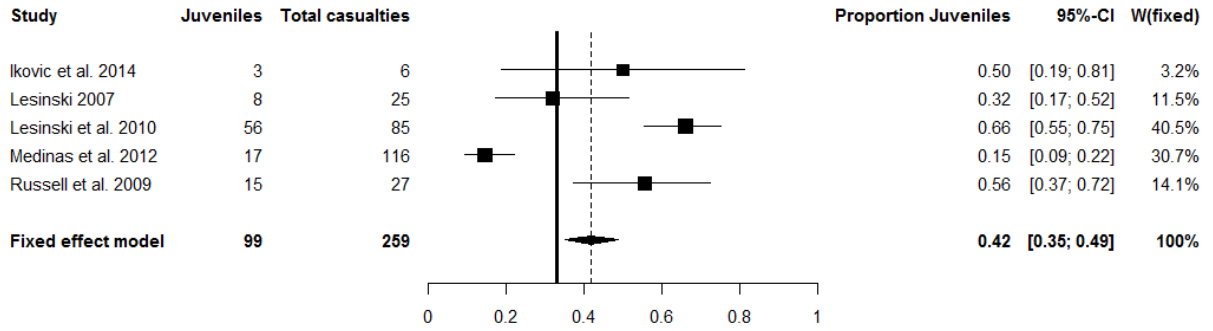
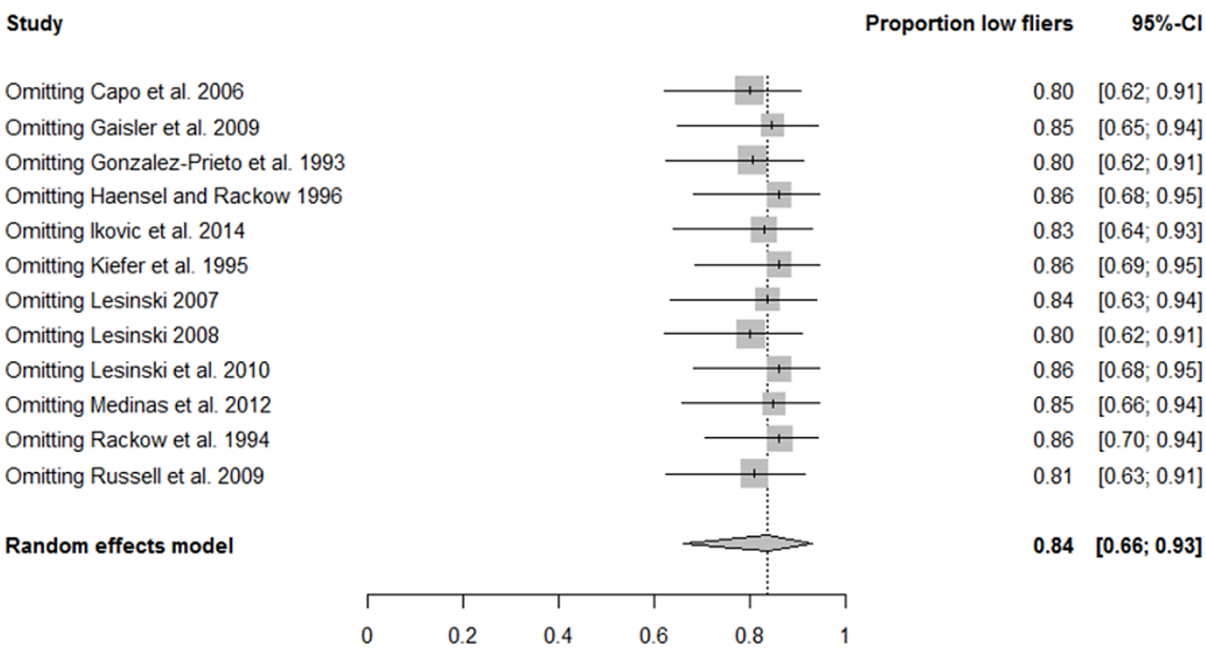
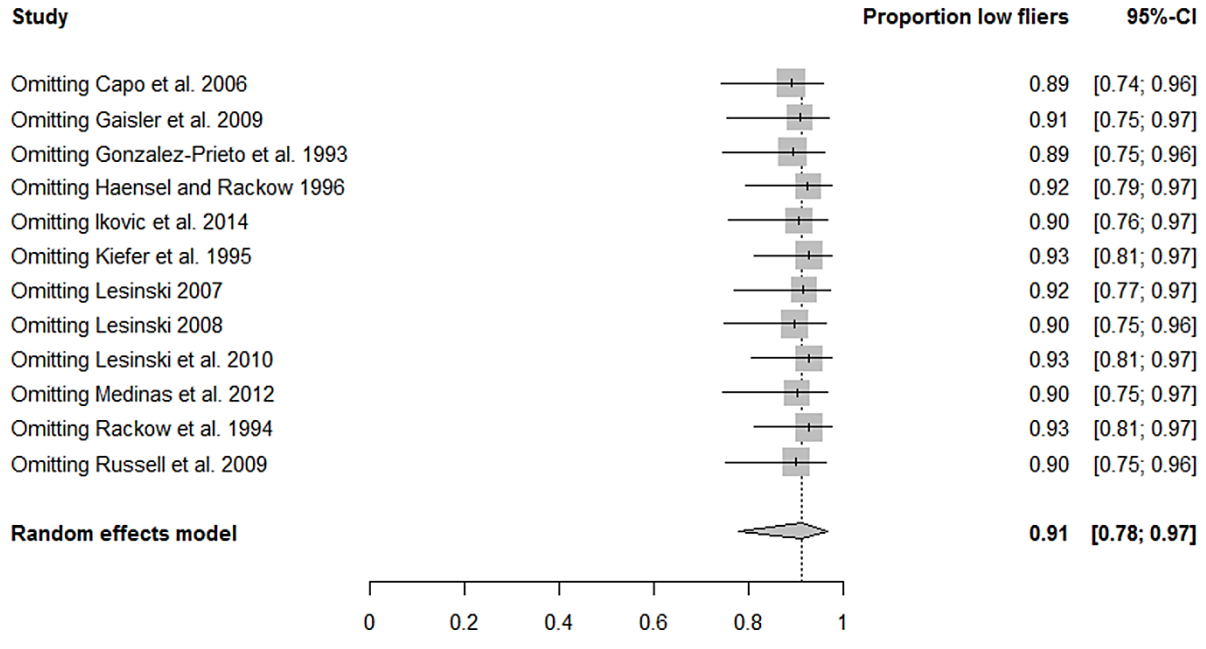


Figure 2.2 Forest plots showing the number of low-flying bats, excluding (a) and including *Pipistrellus* individuals (b), male (c) and juvenile (d) casualties reported in each study, and the overall proportion of each category of casualties obtained from the combined data. A dotted line represents the overall proportion, a solid line represents the expected proportion. Figures 2 a and b do not have a solid line as it was not possible to calculate an expected proportion. The width of the diamond denotes the confidence interval. "W(fixed)" refers to the relative weight of each study under a fixed-effect model.

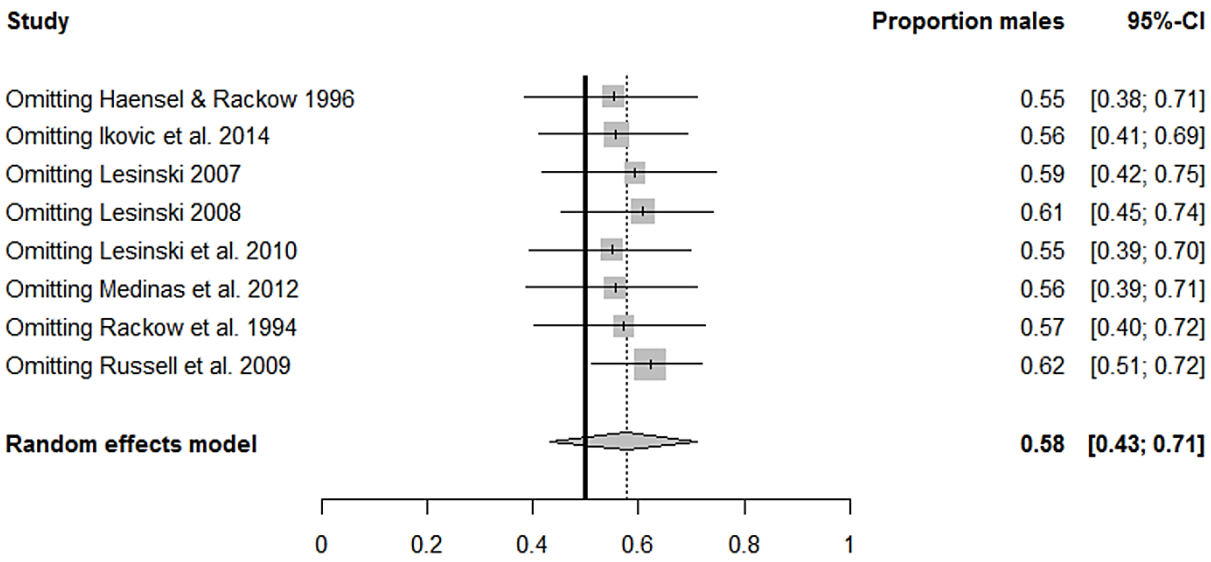
(a)


Study
Proportion low fliers
95%-CI
Omitting Capo et al. 2006
0.80 [0.62; 0.91]
Omitting Gaisler et al. 2009
0.85 [0.65; 0.94]
Omitting Gonzalez-Prieto et al. 1993
0.80 [0.62; 0.91]
Omitting Haensel and Rackow 1996
0.86 [0.68; 0.95]
Omitting Ikovic et al. 2014
0.83 [0.64; 0.93]
Omitting Kiefer et al. 1995
0.86 [0.69; 0.95]
Omitting Lesinski 2007
0.84 [0.63; 0.94]
Omitting Lesinski 2008
0.80 [0.62; 0.91]
Omitting Lesinski et al. 2010
0.86 [0.68; 0.95]
Omitting Medinas et al. 2012
0.85 [0.66; 0.94]
Omitting Rackow et al. 1994
0.86 [0.70; 0.94]
Omitting Russell et al. 2009
0.81 [0.63; 0.91]
Random effects model
0.84 [0.66; 0.93]
0
0.2
0.4
0.6
0.8
1

(b)


Study
Proportion low fliers
95%-CI
Omitting Capo et al. 2006
0.89 [0.74; 0.96]
Omitting Gaisler et al. 2009
0.91 [0.75; 0.97]
Omitting Gonzalez-Prieto et al. 1993
0.89 [0.75; 0.96]
Omitting Haensel and Rackow 1996
0.92 [0.79; 0.97]
Omitting Ikovic et al. 2014
0.90 [0.76; 0.97]
Omitting Kiefer et al. 1995
0.93 [0.81; 0.97]
Omitting Lesinski 2007
0.92 [0.77; 0.97]
Omitting Lesinski 2008
0.90 [0.75; 0.96]
Omitting Lesinski et al. 2010
0.93 [0.81; 0.97]
Omitting Medinas et al. 2012
0.90 [0.75; 0.97]
Omitting Rackow et al. 1994
0.93 [0.81; 0.97]
Omitting Russell et al. 2009
0.90 [0.75; 0.96]
Random effects model
0.91 [0.78; 0.97]
0
0.2
0.4
0.6
0.8
1

(c)



(d)

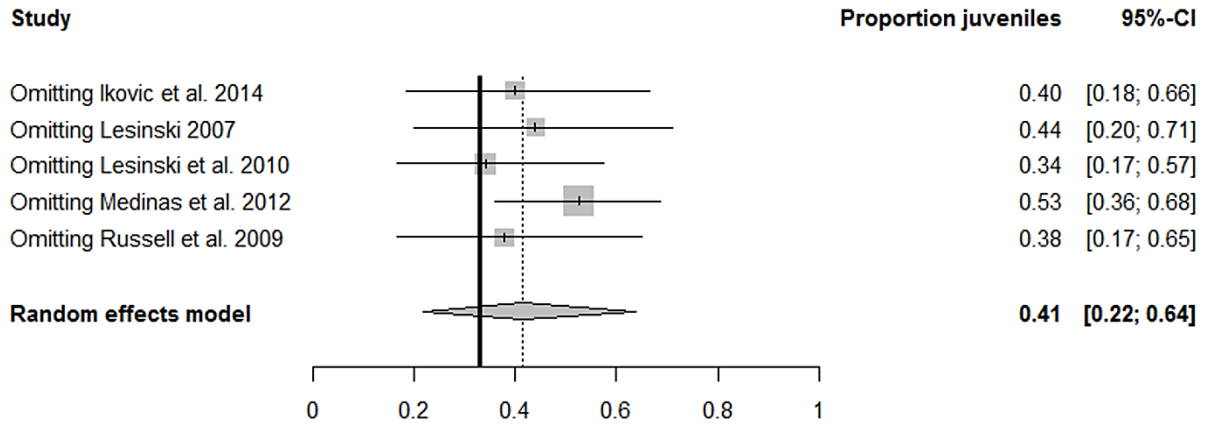


Figure 2.3 Sensitivity analysis for flight height (excluding Pipistrellus; a). Sensitivity analysis for flight height (including Pipistrellus; b). Sensitivity analysis for sex bias (c). Sensitivity analysis for age (d).

2.4.1.2 Environmental risk factors for collision

In seven studies, the authors report that bat casualties were commonly found where roads were close to or bisected other linear features, including tree lines (Lesiński 2008, Russell et al. 2009), hedges (Capo et al. 2006), rivers (Iković et al. 2014), viaducts (Medinas et al. 2012), forest edges and woodland paths (Lesiński 2007, Lesiński et al. 2010; Table 2.6).

There is some evidence that the height and proximity of linear features to roads contribute to collision risk. For example, Russell et al. (2009) note that there were no carcasses where the canopy cover did not run adjacent to the road (within 15 m), but that mortalities were particularly high where the height of canopy declined close to the road. Lesiński et al. (2010) suggest that the relatively high occurrence of *Nyctalus noctula* mortalities found in their study may be due to the intersection of known forest flight paths, the proximity of the woodland to the road and the topology of the site. The combined effect of these ecological attributes may have encouraged *Nyctalus noctula* to cross the road at a lower height than is usual for this species (Lesiński et al. 2010).

The authors of four studies related bat mortalities to the quality of the habitat bordering roads. Medinas et al. (2012) report that bats were more likely to be killed where roads bisect high quality habitats or pass close to scarce and unevenly distributed foraging locations, such as water bodies and riparian habitats. This supports Gaisler et al.'s (2009) and Lesiński's (2007) findings that *Myotis daubentonii* carcasses were found in high numbers near water bodies. Likewise, Iković et al. (2014) found that casualties of *Pipistrellus* species were predominantly clustered at two points where the focal road crossed two tributary rivers.

We proposed that roads could pose a collision risk where they provide foraging opportunities for bats. The systematic review revealed that few researchers have explored this issue. However, the presence of woodland species such as *Barbastella barbastellus* and *Rhinolophus hipposideros* on roads could indicate that they are foraging along roads as they would along woodland paths. Lesiński (2007) suggests that exceptionally high numbers of *Myotis daubentonii* casualties could result from young inexperienced bats mistaking damp road surfaces for the surface of water.

Where a comparison could be made, bat road casualties were more common at locations with greater traffic volume Iković et al. (2014) report that of the 17 bats retrieved from two roads bordered by similar habitat, 16 (0.8/km) were found on the road with relatively high traffic volume (10300 vehicles per day), while just one was found on the road with low traffic volume (1100 vehicles per day). Medinas et al. (2012) report a significant difference in the number of road-killed bats on different types of road: the average number of carcasses found during their survey period was highest (3.99 ± 0.83/km) on the road with the greatest nightly traffic volume (1210 vehicles per night), a little lower (3.60 ± 0.89/km) on a road with intermediate traffic volume (277 vehicles per night), and very low (1.00 ± 0.30/km) on the quietest rural roads (<100 vehicles per night).

Reference	Number of roads	Road length	Road width	Traffic volume	Lit/unlit	Surrounding habitat type
Capo et al. 2006 (France)	1	Not reported	Not reported	Not reported	Partly lit	Sparse vegetation, hedgerow and isolated trees.
Gaisler et al. 2009 (Czech Republic)	2	3.5 km and 4.5 km	Four lane	Not reported	Not reported	One road had a lake on either side, the other crossed over a stream.
Gonzalez-Prieto et al. 1993 (Spain)	1	17 km	Two lanes (10 m wide including hard shoulders)	Not reported	Not reported	Trees, vineyards and shrubland.
Iković et al. 2014 (Montengro)	2	20 km high-traffic road and 10 km low-traffic road	Not reported	One high-traffic road with 10300 vehicles per day and one low-traffic road with 1100 vehicles per day.	One of the monitored sites had streetlights	Meadows, wetlands and Mediterranean karst scrub.
Lesiński 2007 *Method 1* (Poland)	1	8 km	Two lanes	Heavy traffic	Not reported	Not reported
Lesiński 2007 *Method 2* (Poland)	Several	NA	Not reported	Not reported	NA	Not reported
Lesiński 2008 (Poland)	1	1 km	Four lanes	“heavy and round the clock”	Not reported	Allotment gardens on one side and open area on the other, urban development and a section of forest.
Lesiński et al. 2010 (Poland)	1	16.6 km	One lane (7 m)	“relatively intensive”	Not reported	71%, windbreaks and bushes, 3%; built-up area, 4%; meadows and pastures, 15%; and arable fields or wastelands, 7%”

Medinas et al. 2012 (Portugal)	Several	51 km transect	2 lanes on average	Ranged from 1500 per night on busiest road to <100 on quietest roads	Not reported	Primarily Mediterranean agro-forestry.
Russell et al. 2009 (Indiana, USA)	1	4.5 km	20 m wide	8569 vehicles per day (12% trucks)	Not reported	Few buildings and a large area of foraging habitat on opposite side of the road from the roost.

Table 2.6 Site characteristics as reported by authors. This data was not available for three studies (Rackow et al. 1994, Kiefer et al. 1995, Haensel & Rackow, 1996) and so they have not been included.

2.4.2 Barrier effects

2.4.2.1 Risk factors associated with the behaviour and ecology of bats

Meta-analysis reveals that flight height influenced the tendency of bats to avoid roads, as we predicted; some support for the influence of foraging strategy was provided by a qualitative review of the literature (Table 2.7).

Bennett and Zurcher (2013) report that the higher a bat flies as it approaches the road the more likely it is to cross (Table 2.8). *Myotis bechsteinii*, which forages largely by gleaning, was found to be far less likely than *Barbastella barbastellus* (which forages by hawking) to cross a motorway, and only crossed at underpasses (Kerth & Melber 2009). Low flying, clutter adapted *Myotis* spp. and *Plecotus auritus* passed over the road much less frequently than faster, open-edge adapted species (e.g. *Pipistrellus* spp.) or high flying species (e.g. *Nyctalus leisleri;* Abbott et al. 2012), and

752 preferred to use less exposed river bridges and underpasses to cross rather than flying
753 directly over the road (Abbott et al. 2012). Where individuals of *Myotis* spp. and
754 *Plecotus auritus* did cross roads, they preferred following severed treelines to flying
755 over bridges (Abbott et al. 2012). Although species from the genus *Pipistrellus* were
756 more inclined than *Myotis* to use over-road routes, they similarly preferred severed
757 treelines to exposed crossing points (Abbott et al. 2012).

Country	Method	Results	Reference
Ireland	Acoustic monitoring of bat activity at four types of motorway crossing: over road bridges (x6), severed treelines (x6), underpasses (x7) and river bridges (x6). Activity at crossing sites compared to that in adjacent landscape. Road width ~65-70 m, ~20000 vehicles per day	Under-road routes preferred to over-road routes. An average of 23.5 fewer bat passes at over bridges, 7 fewer at severed treelines, 19.5 more passes at underpasses, 158 more passes beneath river bridges than compared to adjacent sites	Abbott et al. (2012)
Ireland	Acoustic monitoring of bat activity at three under road passageways of different dimensions: two narrow (H= 1.23 m, 1.1 m; W=1.48 m, 1.4 m), one wide (H=6 m, W=16.6 m). Road width ~60-65 m, ~11000 vehicles per day	Clutter-adapted species less likely to use over road routes than open or edge-adapted species. Clutter-adapted species were also more likely to use the narrow under road passages	Abbott et al. (2012)
Indiana, USA	Crossing and avoidance behaviour of bats at five survey sites observed. Species identity, flight height, presence/absence of vehicles, and local ecology recorded	Bats were more likely to avoid crossing a road in the presence of vehicles, in the absence of trees, with lower flight height	Bennett & Zurcher (2013)
England	Bat activity monitored acoustically at varying distances (0-1600 m) from a motorway, road width 35 m, 30-40000 vehicles per day	Species diversity and bat activity declined with proximity to the road. Bat activity at 1600 m was 3.5 times that at the road	Berthinussen & Altringham (2012b)
England	Road crossing behaviour monitored acoustically and by observers at four roads to compare frequency with which bats used underpasses, bat gantries and commuting routes. Flight height and verge height flew were also recorded	Bats more likely to cross roads at unsafe heights than to use underpasses. Few bats crossed at gantries but where they did, most flew at unsafe heights (≤5 m). The height at which bats flew over the road was strongly correlated with verge height	Berthinussen & Altringham (2012a)

Germany	Six *Barbastella barbastellus* (low flying and open adapted) and 34 *Myotis bechsteinii* (low flying and clutter adapted) were radio-tracked. Mist-netting conducted in three underpasses. Road width 18-23 m, 84000 vehicles per day	More *Barbastella barbastellus* (5/6) crossed the road than *Myotis bechsteinii* (3/34). Most *Barbastella barbastellus* crossed above the road (21/37); all *Myotis bechsteinii* crossed at underpasses (36/36). *Myotis bechsteinii* foraging ranges were smaller closer to the road and females with smaller foraging areas had lower reproductive success	Kerth & Melber (2009)
California, USA	Bat activity monitored acoustically at three sites, at incremental distances from the road. Road widths and traffic densities: 25-45 m and 55000 vehicles per day; 40 m and 86000 vehicles per day; 15 m and 33500 vehicles per day	Activity was approximately twice as high 300 m from a road as at the road.	Kitzes & Merenlender (2014)
Indiana, USA	Road crossing behaviour (cross/avoid, flight height) at roads was observed at five sites and the presence/absence of vehicles, noise level emitted by vehicles and their speed were recorded	Vehicles present: 40% (28/44) of bats crossed the road. Vehicles absent: 58% (103/167) of bats crossed. Noise level, speed of the vehicle and flight height had no effect on the tendency for bats to cross	Zurcher et al. (2010)

Table 2.7 Articles retrieved during our literature search relating to roads as barriers to the movements of bats.

Vehicles	Height (m)	Crossing
Absent	<7.5	37% (3/8)
Absent	>7.5<14	91% (43/47)
Absent	>14	100% (31/31)
Present	<9	57% (8/14)
Present	>9<13	72% (8/11)
Present	>13	90% (9/10)

Table 2.8 Results reported by Bennett & Zurcher (2013) of the number of bats crossing in the presence and absence of vehicles and the height at which they were observed crossing.

2.4.2.2 Environmental risk factors for barrier effects

The presence or absence of trees and shrub layers were influential factors in determining whether bats crossed roads; the presence of either tended to increase the likelihood of road-crossing behaviour (Abbott et al. 2012, Bennett & Zurcher 2013). Bennett and Zurcher (2013) monitored road crossing behaviour at ten known bat commuting routes bisected by a rural two-lane road, and conducted classification and regression tree analyses to determine which predictor variables, alone or in combination, influenced crossing behaviour in the presence and absence of vehicles. In the presence of vehicles, the classification tree demonstrated a good performance with a reasonable fit, and in the absence of vehicles the model demonstrated excellent performance and a good fit. In both the presence and absence of vehicles, the presence of tree lines was the most influential variable. In the absence of vehicles, 14% (8/57) of bats crossed the road where there was not a treeline, compared to 79%

771 (81/102) where there was a tree line. In the presence of vehicles this effect was more
772 pronounced: just 3.5% (2/58) of bats crossed the road in the absence of a tree line, in
773 contrast to 59% (34/58) in the presence of a tree line (Table 2.9). In the absence of
774 vehicles, the size of the gap between a linear feature and the road was the second
775 most influential variable influencing whether bats crossed or turned back. Fewer bats
776 crossed (2/11,18%) where there was a gap of > 4.5 m than where there were smaller
777 gaps of 2.5 m-4.5 m (2/5, 40%; Bennett & Zurcher 2013).

Vehicles	Tree layer	Crossing
Present	Absent	3.45% (2/58)
Present	Present	58.62% (34/58)
Absent	Absent	14% (8/57)
Absent	Present	79% (81/102)

Table 2.9 Results reported by Bennett & Zurcher (2013) of the number of bats crossing in the presence and absence of vehicles and the presence or absence of a tree layer.

778 At two under-road crossing points (underpasses and river bridges), high
779 numbers of bats passed above the road, which the authors attributed to the presence
780 of extensive tree canopies above the motorway at these locations (Abbott et al. 2012).

781 Direct evidence for the influence of road width on crossing behaviour is lacking.
782 However, two studies, one from the UK (Berthinussen & Altringham 2012b) and one

783 from the USA (Kitzes & Merenlender 2014), suggest that bat activity declines with
784 proximity to major roads. Bat activity was 3.5 times higher at a distance of 1600 m
785 from a 6-7-lane road (approximately 32 m wide) than at the road (Berthinussen &
786 Altringham 2012b), and activity was twice as high at 300 m from a 2-lane road (15 m
787 wide; Kitzes & Merenlender 2014).

788 The authors of two studies explored the impact of traffic on the probability of
789 road crossing. Bennett and Zurcher (2013) found that in the absence of vehicles, 56%
790 (89/159) of bats aborted crossing attempts, but in the presence of vehicles this rose
791 to 68% (74/107); it rose further still to 100% (34/34) if vehicles produced noise levels
792 above 88 dB. Bats may be particularly sensitive to noise pollution because they use
793 echolocation to hunt insect prey and to orientate themselves in their environment;
794 *Myotis myotis* has been shown to avoid foraging in proximity to loud noise (Schaub et
795 al. 2008), and foraging efficiency has also been observed to decline with proximity to
796 traffic noise (Siemers & Schaub 2011). Zurcher et al. (2010) found that 32% (64/167)
797 of bats aborted crossings in the absence of vehicles compared with 60% (29/44) in the
798 presence of vehicles. It is unclear whether this effect is due to the influence of noise,
799 vehicle headlights, or both.

800 2.5 Discussion

801 We have identified substantial evidence indicating a significant risk to bats from
802 roads, particularly through collision risk.

803 Our data indicate that casualties are more likely to be low- than high-
804 flying. Determining the likely impacts on populations is difficult in the absence of good
805 data on population density. In Great Britain, the best available estimate indicates a
806 ratio of low-: high-flying bats of 0.97 including pipistrelles and 0.89 excluding
807 pipistrelles (Harris et al. 1995, Battersby 2005). If these proportions are representative
808 of mainland Europe, the casualty ratios of 0.73 (including pipistrelles) and 0.64
809 (excluding pipistrelles) found in this project suggest that whilst low-flying species are
810 the most common casualties, high-flying bats are more frequently hit than would be
811 expected from their relative population sizes.

812 A number of rare species such as *Barbastella barbastellus* and geographically
813 restricted species such as those from the genera *Rhinolophus* and *Plecotus* were also
814 found on roads. The presence of casualties from rare species on roads is of particular
815 concern, as relatively low levels of additional mortality could potentially have an impact
816 on the long-term sustainability of local populations.

817 Meta-analyses confirm significant biases towards juvenile and male casualties
818 on roads. The higher number of male fatalities could be due to female-biased
819 philopatry and male-biased dispersal, which are typical of mammal breeding systems
820 (Greenwood 1980). Greater dispersal distances could mean that males encounter
821 roads more often, and inexperienced sub-adult males may be at particular risk. Males
822 may also be more susceptible to collisions if they are more likely to roost or forage in
823 the vicinity of roads: in many species, there is sexual segregation during the breeding
824 season, and some evidence suggests that female bats occupy better quality habitats
825 (Angell et al. 2013) or less fragmented habitat (Lintott et al. 2014) during this period.

826 If and where roads represent, or are associated with, poor quality habitat (i.e. due to
827 edge effects), it is possible that females are restricted to areas with lower road density.

828 Although our meta-analysis shows that overall males have a higher casualty
829 risk, in some locations there was excess mortality in females. This may be due to the
830 proximity of a particular road to a maternity roost. Medinas et al. (2012) report male
831 bias overall, but note that during early summer twice as many female as male
832 carcasses were found on the roads, corresponding with the time of year when females
833 form maternity roosts. The formation of maternity roosts in early summer could explain
834 the second highest peak in mortalities which occurred in May (Figure 2.1).

835 We identified considerable support for the hypothesis that the risk of collision
836 increases at junctions between roads and other linear habitat features. Tree lines
837 running perpendicular to roads are preferred crossing points (Abbott et al. 2012,
838 Bennett & Zurcher 2013), and the proximity of tree stands and tree lines to roads
839 appears to increase the propensity of bats to cross roads (Bennett & Zurcher 2013).
840 These features are also associated with mortality hotspots (Capo et al. 2006, Lesiński
841 2007, Lesiński 2008, Lesiński et al. 2010, Russell et al. 2009).

842 Unfortunately, it is difficult to draw firm conclusions about the effects of fatalities
843 on local populations as they are rarely quantified. However, even low adult mortality
844 may reduce effective population size in bats (Schorcht et al. 2009). This is particularly
845 worrying given the presence of geographically restricted and locally vulnerable species
846 such as *Plecotus auritus*. It is likely that the numbers reported underestimate true
847 collision rates, as none of the researchers adjusted the observed casualty rates for
848 observer efficiency and the removal of carcasses by predators. Removal or destruction
849 of carcasses may significantly bias results, as carcasses do not persist for long on

roads (Santos et al. 2011). For comparison, casualty rates at wind turbine sites are often considerably higher than the numbers of observed carcasses (see Huso 2011, Bernardino et al. 2012, Bispo et al. 2013).

Additional research is required to understand fully the factors influencing road-crossing behaviour in bats. Most species have been shown to cross roads (Tables 2.4 and 2.7 and references therein), but clutter-adapted species, i.e. those species that are adapted to navigating woodland edges and interior, are least likely to do so (Abbott et al. 2012, Bennett & Zurcher 2013, Kerth & Melber 2009). Where passages under roads are available, these are preferred to over-road routes (Abbott et al. 2012). Major roads appear to be more inhibitive than secondary roads (Berthinussen & Altringham 2012b, Kitzes & Merenlender 2014), perhaps as a result of associated high traffic volumes on major roads (Bennett & Zurcher 2013). There is some evidence that roads with greater traffic densities are associated with higher collision rates between bats and vehicles (Ikvić et al. 2014, Medinas et al. 2012). This apparent contradiction could be resolved with a better understanding of how different species respond to traffic, and perhaps more consistent reporting of traffic volume data.

Whether and how a particular road poses a threat to bats is species-dependent; a given road could simultaneously pose a threat to some species as a result of collisions, and form an impermeable barrier to movement for other species. Some species' characteristics may increase the probability of both collision and barrier effects. For example, clutter-adapted bat species are predicted to avoid roads, as they are associated with closed environments such as woodland interior. However, these species may sometimes commute or forage in small open spaces. It is possible that such species cross roads where the roads are narrow or where they are bordered by

874 trees. The slow and low flight of clutter-adapted species puts them at greater risk of
875 collision with vehicles. Therefore habitat fragmentation and collision risks may act in
876 combination for some species.

877 A key challenge for bat conservation is resolving how to increase the
878 permeability of roads to bats without increasing the likelihood of vehicle collision.
879 Gantries are often included in the mitigation design of new roads. These structures
880 span the road and, where possible, link linear traditional commuting features used by
881 bats on either side of the road. Recent research suggests, however, that the few bats
882 observed crossing at gantries do not increase the height at which they cross the road
883 (Berthinussen & Altringham 2012a). Green bridges and under-road passages should
884 be explored as alternative forms of mitigation. Further research needs to be conducted
885 on the efficacy of gantries and underpasses in relation to wider roads. Specifically,
886 there is a need for more surveys of bat activity before and after road construction.
887 Even where pre- and post-construction surveys are conducted, different protocols and
888 recording equipment are often used, and surveys often take place over a short period,
889 making adjustments for the effects of factors such as weather extremely challenging.

890 Due to a lack of data, we were unable to investigate the influence of road age
891 on collision risk or crossing behaviour of bats in this study. However, the age of a road
892 could influence how bats interact with it, through either sensitisation (resulting in more
893 pronounced avoidance behaviour and reduced collision risk over time) or habituation
894 (more frequent crossing attempts and increased collision risk over time). The threat
895 posed by a road could also appear to decline over time where local abundance of bats
896 becomes suppressed as a result of collisions. The influence of road age on collision

risk and crossing behaviour should be examined by means of long term, systematic pre- and post-construction studies, where new roads or road adjustments are planned.

Several species of bat are known to avoid artificial lighting (Day 2017). The influence of artificial lighting is likely, therefore, to be one of the most significant factors determining how bats make use of landscapes, and whether roads present a barrier to movement (Hale et al. 2015, Mathews et al. 2015). To understand the relative influence of road characteristics on species' responses, future research should compare the effects on bats of roads that are lit with those that are unlit.

Bats are much less likely to cross roads in the presence of particularly loud vehicles (Bennett & Zurcher 2013). Further research is needed to understand the extent to which noise, specifically volume and frequency, effects road-crossing behaviour. It is also possible that disorientation as a result of traffic noise could make bats susceptible to collisions.

The need to examine the impact of roads is pressing; road density is already extremely high in the UK and mainland Europe, and further development is expected in the near future. To ensure that appropriate and effective mitigation is incorporated during the planned period of intensive road expansion, it is essential that the factors influencing road crossing behaviour in bats are fully understood. A better understanding of how bats interact with roads of varying widths, traffic densities and lighting schemes, as well as the role of the surrounding topography and habitat, could contribute to the design of safe crossing points.

Chapter 3: Preserve, connect, improve: Area-dependent occupation of road-fragmented patches by bats in the UK

Thesis pp 121 – 164

3.0 Preserve, connect, improve: Area-dependent occupation of road-fragmented patches by bats in the UK

3.1 Summary

Human-altered environments now extend over 75% of the Earth's terrestrial surface. A conspicuous way in which humans have changed landscapes is through the construction of road networks. For some species, roads can act as a partial or complete barrier to landscape-scale movement. Bats may be particularly vulnerable to the barrier effect of roads as they have large habitat requirements and can travel considerable distances; for example, between winter and summer roosts. Major roads may be a more impermeable barrier to bats than minor roads as they are wider, and often have a greater density of traffic, less bordering vegetation and are more likely to be artificially lit. Minor roads represent direct habitat loss and may be associated with edge effects. By acting as a barrier to movement, major roads could determine the availability of accessible resources. This being so, and given the large habitat requirements of bats, it is possible that bats are more likely to roost in larger patches as defined by major roads. Here, I test whether the distribution of bat roosts in the UK is related to the size of patches delineated by major roads (PDMRs). I also examine the influence of patch quality including the density of minor roads, and the area of woodlands, grassland and built environments. To determine whether road-defined patches were occupied by at least one roost I used the locations of 6199 bat roosts obtained from Natural England and the National Bat Monitoring Programme (NBMP). My analyses demonstrate that bats are more likely to be found to roost in larger

patches (PDMRs) and that the patches occupied by roosts have a greater density of minor roads, and a greater areal cover of broadleaved woodland and built environments than unoccupied patches, but less coniferous woodland. My results suggest that conservation of bat populations could be improved if road-defined patches are preserved, connected and improved. Consideration must be given to increasing connectivity through means that do not increase the risk that bats are killed by collisions with vehicles.

3.2 Introduction

Anthropogenic transformation of land cover from natural vegetation to farmland and built environments extends over approximately 75% of the Earth's terrestrial surface (Ellis & Ramankutty 2008). The hallmarks of human-exploited landscapes include the loss, fragmentation and degradation of natural habitats due to urban expansion, conversion to agricultural land, resource extraction, and the construction of transport infrastructure, including road networks (Brooks et al. 2002; Ellis 2011; Haddad et al. 2015). The road network is particularly dense in parts of North America and through most of Europe, including the UK (Torres et al. 2016; Ibisch et al. 2017). Road density across Europe is expected to increase by 2020 as the European Commission is committed to investing €500 billion in the transport network (Europa.eu, 2017) and the UK government alone plans to spend £15 billion on new roads and road widening schemes (Department for Transport 2014) over the next decade.

For wild populations that live in landscapes permeated by road networks, abundances of individuals declines with proximity to roads in a wide range of taxa that includes amphibians, reptiles, mammals and birds (Fahrig & Rytwinski 2009; Benítez-

963 López et al. 2010; Karlson & Mörtberg 2015; Torres et al. 2016; Galantinho et al.
964 2017), which suggests that roads can threaten overall biodiversity. Some of the effects
965 on populations may be explained by road-related mortality (Fahrig & Rytwinski 2009),
966 which is extensive amongst amphibians (Fahrig et al. 1995; Glista et al. 2008; Hels &
967 Buchwald, 2001), reptiles (Chittaragi & Hosetti, 2014; Heigl et al. 2017; Sosa & Schalk,
968 2016), birds (Erritzoe et al. 2003; Kociolek et al. 2011), invertebrates (Shyama Prasad
969 Rao & Saptha Girish 2007) and mammals (Langbein 2011; Fensome & Mathews
970 2016).

971 Roads can also have indirect impacts on population abundance and
972 persistence by reducing the availability, accessibility and quality of the habitats in their
973 vicinity (Trombulak & Frissell 2001; Coffin 2007). For example, roads not only occupy
974 previously habitable area, but they also facilitate the encroachment of human
975 developments leading to further loss and degradation (Trombulak & Frissell 2001;
976 Coffin 2007). Roadside habitats can be considerably altered by chemical pollution from
977 vehicle emissions, changes to light and temperature regimes caused by streetlighting,
978 and disturbance by artificial lighting and traffic noise (Trombulak & Frissell 2001; Coffin
979 2007) producing edge effects around roads that can exert an influence up to 5 km from
980 the curb of the road (Benítez-López et al. 2010; Ibisch et al. 2017).

981 For some animal species, roads act as a partial or complete barrier to
982 movement (Coffin, 2007; Trombulak & Frissell, 2001), thereby creating landscapes
983 that appear to be geographically continuous but that are in actuality ecologically
984 fragmented. By fragmenting landscapes into patches, roads could have profound
985 ecological impacts. For species that avoid roads, patch size (i.e. the area of road-
986 delineated patches, or RDPs) could determine the availability of accessible resources

and hence influence the viability of resident populations (Jaeger et al. 2005; Eigenbrod et al. 2007). Restrictions on patch size caused by roads may be particularly problematic for species with large home ranges (Fahrig & Rytwinski 2009; Torres et al. 2016) and species dependent on interior habitats (as patch size decreases, the area of core habitat relative to edge habitat decreases; Bender et al. 1998). By acting as a barrier to movement, roads could prevent recolonization of depopulated fragments (Forman & Alexander 1998) or reduce gene flow between isolated populations (Holderegger & Di Giulio 2010).

Bats may be particularly vulnerable to landscape fragmentation caused by barrier effects of roads. Bats may avoid crossing roads (Fensome & Mathews 2016), particularly in species that are low-flying and/or adapted to foraging in woodland environments (i.e. clutter-adapted species; Appendix 2.1). The impacts of fragmentation may be severe for bats, which often require relatively large areas for foraging; UK bats are central-place foragers around their roost and they can exploit an area of between 3 km^2 and 50 km^2 depending on the species (Bat Conservation Trust UK 2016), which is termed a 'core sustenance zone'. Roads also may constrain movement of bats generally. Roads could severely restrict access to traditional roosting, mating and foraging sites and limit dispersal to new sites (Voigt & Kingston 2015). Roads may also disrupt bats use of linear features (e.g. hedgerows and field edges) to navigate through the landscape and so bats are sensitive to disruption to landscape connectivity (Voigt & Kingston 2015).

Major roads are potentially a more effective barrier to bats than minor roads. Major roads tends to be wider than minor roads (Hansard 2003); in the UK, motorways have at least four lanes, "A" roads have at least two lanes, whilst minor roads are often

a single lane. Several species of bat avoid flying in open spaces, which suggests wider roads could be less permeable as boundaries to flying bats than narrow roads (Kerth & Melber, 2009; Voigt & Kingston, 2015). Traffic density is also considerably higher on major roads than minor roads; in 2016, 29.47 million vehicle miles were travelled on motorways compared to 4.94 million on “A” roads and just 0.52 million on minor roads (Department for Transport 2017a, 2017b). There is evidence that bats are less likely to cross roads in the presence of traffic (Zurcher et al. 2010; Bennett & Zurcher 2013), especially loud traffic (Zurcher et al. 2010). Several species of bat avoid artificial lighting (Stone et al. 2015; Table 1.2) which may exacerbate the barrier effect on busy, urban roads (Voigt & Kingston 2015). A combination of these factors may explain why bat activity has also been observed to dramatically decline with proximity to motorways (Berthinussen & Altringham 2012a; Kitzes & Merenlender 2014).

By contrast, minor roads may be less likely to restrict the movement of bats through the landscape (Fensome & Mathews, 2016; Voigt & Kingston, 2015). In fact, the density of minor roads could be a proxy for a combination of measures related to the quality of a patch with either positive or negative connotations. on the one hand, minor roads represent direct habitat loss and may be associated with detrimental edge effects (Spellerberg 1998). As road density increases, for example, the amount of road-free area will decrease as habitat becomes increasingly fragmented (Ibisch et al. 2017). We might therefore predict that as minor road density increases, the likelihood of a roost being present within a patch decreases as a result of diminishing patch quality. On the other hand, minor roads are often bordered by trees and hedges, especially in rural areas. It is possible therefore, that minor roads offer foraging opportunities for bats that compensate the direct habitat loss and edge effects associated with minor roads. We might therefore predict that as minor road density

increases, the likelihood of a roost being present within a patch increases as a result of increasing patch quality.

If major roads are impermeable or partially permeable barriers to landscape-scale movement, then local populations may be affected by the restriction of their activity within a given fragment. In these cases, the impact of the roads on bats are mediated by the quality of the area of accessible habitat delineated by roads. I therefore examine the influence of several measures of patch quality including the area of broadleaved and coniferous woodlands, grassland, and built environments within patches, each of which might be positively or negatively associated with different kinds or sizes of roads. Each of these types of habitats provide foraging and/or roosting opportunities, including built environments (Russ 1999; Voigt & Kingston 2015).

Even so, the overall impact of these associations between road-delineated patches and the kinds of habitats they enclose is not clear *a priori*. For example, bats can benefit from the presence of built environments because several species of bat are known to roost in houses and outbuildings (The Bat Conservation Trust 2012). However, bats could be excluded from patches with dense built environments due to either a lack of adequate foraging habitats such as woodland and grassland or the presence of more artificial lighting. or a combination of all of these factors (Oprea et al. 2009; Hale et al. 2015; Russo & Ancillotto 2015). Although bats readily exploit human dwellings, generally urbanization has a negative effect on bats (Voigt & Kingston 2015). Several studies have demonstrated that species diversity declines with increasing urban influence (Voigt & Kingston 2015). Bat activity has also been shown to decline as measures of urbanisation, such as built environment cover and

population density, increase (Hale et al. 2012; Border et al. 2017). Hale et al. (2012) were able to demonstrate that activity declines non-linearly with the percentage of built land-cover. Activity was relatively high between 0 and 20% built land-cover, peaked at 40% and rapidly declining thereafter. Areas with 80% built land-cover had less than half the activity of areas with 0-20% built land-cover.

In summary, the objectives of our study were to test the hypothesis that bats are influenced by the topography of road networks. I studied the relationships between road-delineated habitat patches and the roost distributions of 17 species from the following genera: *Barbastellus* (1 species), *Eptesicus* (1), *Myotis* (7), *Nyctalus* (1), *Pipistrellus* (3), *Plecotus* (2), *Rhinolophus* (2). Specifically, I investigated whether the distribution of bat roosts in the United Kingdom is related to the size of patches created among intersecting roads. Additionally, I relate patch occupancy to attributes of those patches such as the density of minor roads and the availability of useful foraging and roosting habitat types within patches.

3.3 Methods

3.3.1 Creating the dataset

I divided the UK into all possible patches that were delineated by major roads (A and B-roads, and motorways) in ArcGIS (ESRI v 10.3 2014). I calculated the area (km^2) of these patches as well as the density of minor roads (m/m^2) within them. The areas of broadleaved and coniferous woodland, grassland and built environments (suburban and urban built areas) within each patch were measured using the Centre

for Ecology and Hydrology's (CEH) Land Cover Map 2007 (LCM 2007, Morton et al. 2011).

In order to determine patch occupancy, I used bat roost locations obtained from the National Bat Monitoring Programme (NBMP) dataset (with permission from the Bat Conservation Trust) and Natural England. Roosts were selected from the datasets if the location was precise to within 10-100m^2. A total of 6199 roosts from 16 species (*Myotis bechsteinii* was absent from the dataset; Appendix 3.1) were selected for this study. Each roost was assigned to a patch. In total, the UK was divided by major roads into 13893 patches, which were either unoccupied (n=12,054) or occupied (n=1839; for breakdown by country see Appendix 3.2) by at least one roost of at least one species of bat. As required, the total area of these patches combined was equal to the total area of the UK mainland (219,641 km^2).

3.3.2 Analysis

3.3.2.1 Aggregated quartiles

In order to investigate the relationship between patch size and roost presence, patches were ranked by size (km^2) before being aggregated into four groups, or 'area quartiles' that each contained 25% of the UK's land area. The first group was constituted by the area quartile comprising the smallest fragments (n= 12601), the second and third groups consisted of intermediate sized fragments (n= 863 and n= 357 respectively), whilst the fourth group contained the area quartile of the largest fragments (n=72). As required, the combined area (km^2) of patches in each group was approximately one quarter of the area of the UK mainland (median 54,899 ± 77 km^2).

In effect, I created four hypothetical landscapes of equal area but with different degrees of fragmentation; quartile 1 represents the most fragmented landscape (because in aggregate it comprises the smallest road-bounded fragments) and quartile 4 represents the least fragmented landscape, being composed of the largest fragments. By making the unit of comparison the landscape rather than the patch, I was able to control for an inherent bias of directly comparing the mean patch size (and the areas of habitats within them) of occupied and unoccupied patches: as patch size increases, the likelihood of it containing a roost, and greater amounts of any type of habitat, naturally increases.

3.3.2.2 Abundance

I compared the number of roosts contained within each quartile. The total number of roosts was 6192 after excluding roosts without species identification (n=7). If there was no effect of roads on the presence of bat roosts, we would expect each quartile to contain the approximately same number of roosts (n=1548) subject to appropriate sampling error. Because the data are frequency counts and the comparison is against a set of expected frequencies (i.e. H0: even representation of bat roosts), I used a Pearson's chi-squared goodness-of-fit test to determine whether there was a significant difference between the observed and expected number of roosts in each quartile.

3.3.2.3 Prevalence

I compared the proportion of patches occupied by at least one roost within each quartile and used a Pearson's chi-squared test to determine whether there was a significant difference between the observed and expected proportion of occupied patches in each quartile. If there was no effect of patch size determined by roads, we would expect each quartile to contain the same relative frequency of occupied patches (in practice, the expected number of occupied patches varies among the area quartiles because they contain different numbers of patches). The expected proportion of occupied patches in each quartile is the total number of occupied patches in all quartiles (n=1839) divided by the total number of occupied and unoccupied patches (n=13893) and this expected frequency in each area quartile is obtained by multiplying this proportion by the number of patches in the quartile. I used a Pearson's chi-squared goodness-of-fit test to determine whether there was a significant difference between the observed and expected numbers of occupied patches in each quartile.

3.3.2.4 Average size of patches containing zero, one or multiple roosts within quartiles

Within each area quartile, I compared the average size of road-defined patches by Analysis of Variance (ANOVA) to determine whether there was a significant difference between patches containing zero, one or multiple roosts.

3.3.2.5 Patch quality

I compared the quality of occupied and unoccupied patches within each area quartile by comparing the mean areas of broadleaved and coniferous woodland, grassland and built environments as well as the density of minor roads. I tested whether the mean areas of each habitat type were significantly different in occupied and unoccupied patches with a Wilcoxon rank-sum test.

3.3.2.6 Regression analysis

In order to further explore the influence of patch size and patch quality on the presence of roosts within patches prior to GLM, I binned patches in incremental categories of 5% for broadleaved woodland, coniferous woodland, grassland and built environments and in increments of 5 km^2 for patch area and of 2 km/km^2 for minor road density. I plotted the proportion of occupied patches in each bin against bin size and calculated the R^2.

3.3.2.7 Multi-model inference and a Binomial Generalized Linear model

In order to compare the relative influence of patch size and habitat attributes on the probability of a roost being present within a patch, I constructed a binary logistic regression model. I screened all potential variables for inclusion with univariate logistic regression with a relaxed inclusion criterion ($p < 0.1$). I used an information theoretic approach to model selection (Burnham & Anderson 2010). During model selection, the relative likelihood of the increasingly restrictive models was compared at each step using Akaike's information criterion (with each simplification accepted provided that

delta AIC ≤ 4) (R v. 3.1.0, MuMIn package). I calculated the odds ratio and confidence intervals for each variable in the most supported model. From the final binomial model, I was able to generate a probability curve for patch size and to calculate the effect of splitting a patch of a given size into two equal halves; i.e. the combined probabilities of a roost being present within two new road-defined patches compared to the original probability of a roost being present in the un-bisected road-defined patch.

3.3.2.8 Exploring robustness and generality across bat species

Taxonomic comparisons

Analyses were repeated for subsections of the dataset that each comprised an individual species (where sample size allowed) or genus (*Pipistrellus* and *Myotis*) in order to assess the generality of the primary analysis. *Plecotus auritus* (roosts, n=1261; occupied patches, n=692), *Rhinolophus hipposideros* (roosts, n=483; occupied patches, n=233), and *Eptesicus serotinus* (roosts, n=225; occupied patches, n=154) had sufficiently large sample sizes to conduct these separate analyses.

Roosts belonging to species within the genus *Pipistrellus* were combined (roosts, n=3561; occupied patches, n=1606). This was to account for the fact *P. pipistrellus* and *P. pygamaeus* were considered one species until the 1990s. It is possible that some *P. pygmaeus* roosts were misidentified as *P. pipistrellus* roosts. Additionally, a number of roosts had been identified to the genus level only (n=517).

Species from the genus *Myotis* were combined (combined roosts, n=538; occupied patches, n=311) for two reasons. First, to avoid any uncertainty about the accuracy of species' identity; *Myotis* species can be very difficult to identify visually or

in the hand and extremely difficult to differentiate acoustically. Second, some species had very few records (e.g. *Myotis alcathoe*, n=2).

Combining species within the genera *Pipistrellus* and *Myotis* was also justified as species within their respective genus share traits that predict road crossing behaviour. *Myotis* species are low-flying, clutter-adapted and avoid artificial lighting whereas species in the genus *Pipistrellus* are more variable in their flight height and foraging habits and less sensitive to artificial lighting.

The influence of urbanisation

In order to test the hypothesis that the influence of patch size on the abundance and prevalence of roosts and the influence of patch quality on the selection of patches did not solely reflect avoidance of densely urbanised patches, I repeated the analyses of abundance, prevalence and patch quality on three subsets of data with patches that contained 0-80%, 0-20% and 80-100% built environments within patches. For example, a greater abundance or prevalence of roosts in aggregates composed of larger patches, could simply reflect avoidance of highly urbanised patches which are likely to be smaller.

I created three subsets of data. The first contained patches (occupied and unoccupied) with all but the most urbanised patches (0-80% built environments; n=8737). The second subset dataset contained only the least urbanised patches, i.e. those with less than 20% built environments (0-20%; n=5118) whilst the third dataset contained the most urbanised patches (80-100%; n=5156).

Sampling bias

Sampling bias likely exists in the datasets from which I obtained roost locations. Following analysis, it became evident that larger road-defined patches tended to have a lower density of minor roads and a lower proportion of built environments, and are by definition, remote. They also have the greatest proportion of woodlands. It is possible that roosts in patches with these qualities are less likely to be encountered by people. I discuss the potential impact of this bias on the results in the discussion.

All analyses were carried out in R Statistical package (R Core Team 2014).

3.4 Results

Major roads fragment the UK into 13893 patches that range in size from less than one square meter to 2790.27 km^2. The majority of patches were very small; 63% of patches were less than 3 km^2 and 69% were less than 5 km^2. Scotland has a greater proportion of the largest patches: 58% of patches in the largest aggregated group are in Scotland and 30% are in England. In the smallest aggregated group 81% of fragments were in England and 12% in Scotland (Figure 3.1).

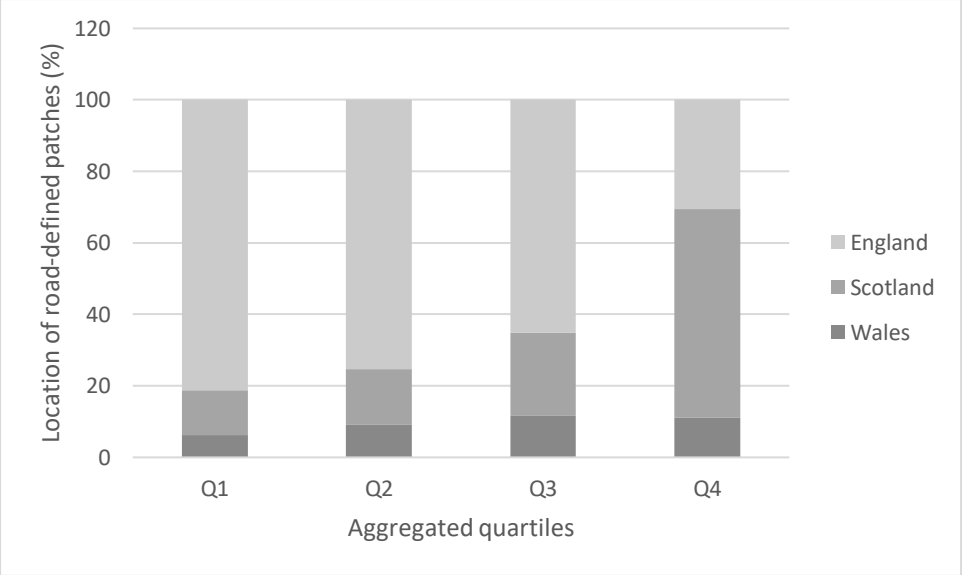


Figure 3.1 The percentage of patches in each aggregated quartile located in England, Scotland and Wales.

3.4.1 Representation of roosts among area quartiles

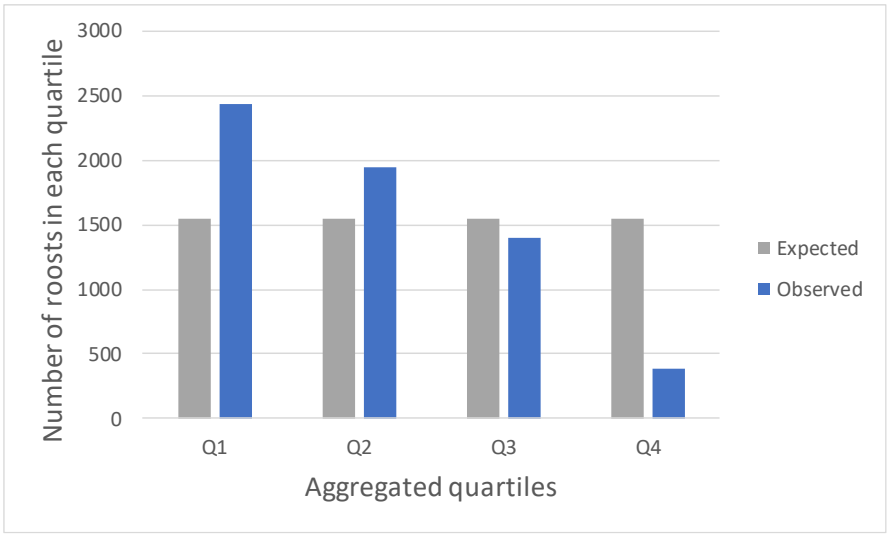
3.4.1.1 Abundance

More roosts were found in the quartile composed of the smallest patches than expected and fewer roosts were present in the quartile composed of the largest patches (Figure 3.2 a; Table 3.1).

3.4.1.2 Prevalence

The proportion of occupied patches in each aggregated quartile increased as patch size increased (Figure 3.2 b; Table 3.1).

(a)



(b)

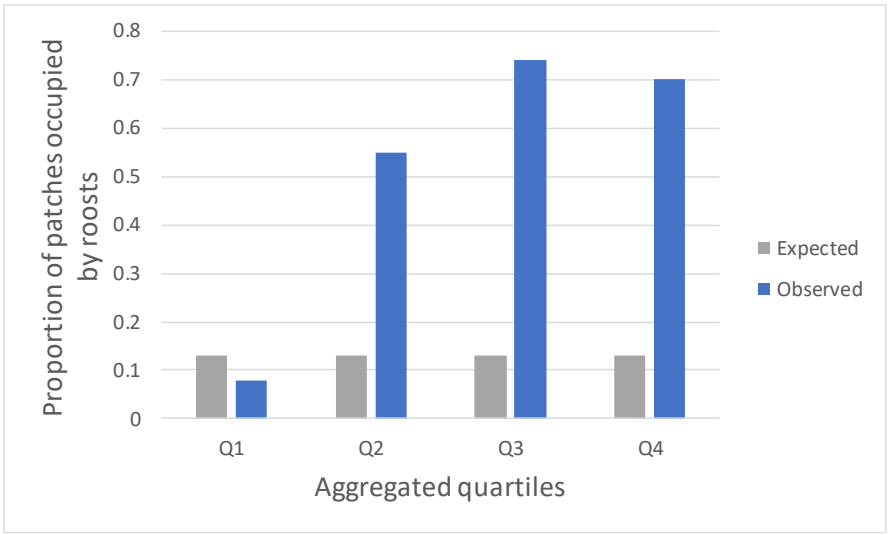


Figure 3.2 The expected and observed number of roosts in each aggregated quartile (a) and the expected and observed proportion of road-defined patches occupied by at least one roost (b).

The number of roosts in each quartile					
Figure 3.2		**Expected**	**Observed**	**χ^2**	**p-value**
(a)	Quartile 1	1548	2443	1552.15	<0.001***
	Quartile 2	1548	2010		
	Quartile 3	1548	1353		
	Quartile 4	1548	386		

The proportion and number of occupied patches in each quartile							
		Proportions		**Frequencies**			
		E	**O**	**E**	**O**		
(b)	Quartile 1	0.13	0.08	1667	1044	2586.27	< 0.001***
	Quartile 2	0.13	0.55	115	484		
	Quartile 3	0.13	0.74	45	260		
	Quartile 4	0.13	0.70	9	51		

Table 3.1 The expected and observed number of roosts (a) and proportion of occupied patches (b) for each aggregated quartile and the results of Pearson's chi-squared tests.

3.4.1.3 Occupied patch area

The summed area of patches containing multiple roosts increased across the four quartiles (i.e. the total area of the occupied fragments was greatest in the area quartile that was comprised of the largest patches.) whilst the summed area of patches containing no roosts decreased across quartiles. The summed area of patches containing one roost was similar across quartiles (Figure 3.3).

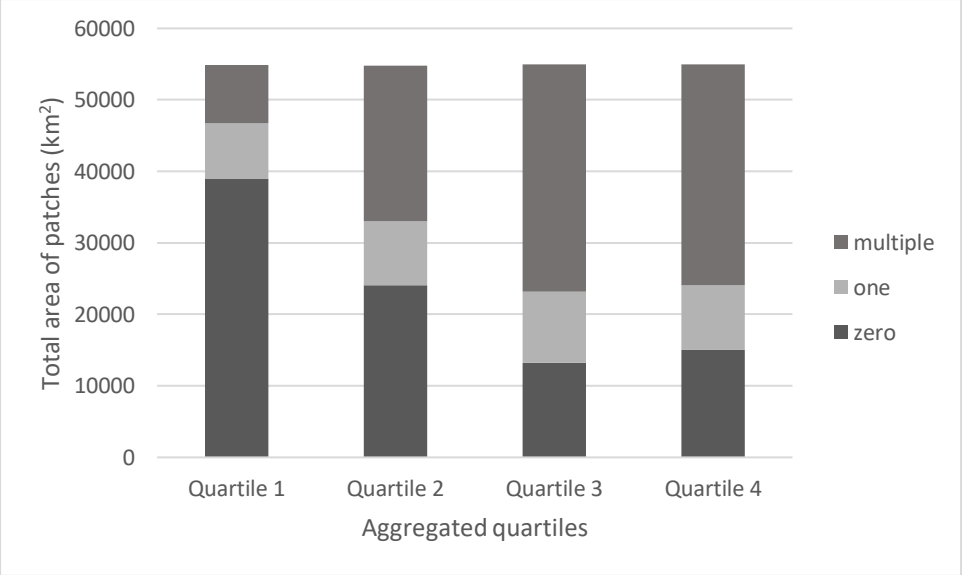


Figure 3.3 The combined area (km^2) of road-defined patches containing zero, one or multiple roosts in each aggregated quartile.

Summed area of road-defined patches (km^2)				
	Zero roosts	**One roost**	**Multiple roosts**	**Total**
Quartile 1	38951	7797	8158	54906
Quartile 2	24032	9018	21770	54821
Quartile 3	13247	9966	31762	54976
Quartile 4	15022	9075	30839	54936

Table 3.2 The summed area of patches that contain zero, one or multiple roosts.

3.4.1.4 Average size of patches containing zero, one or multiple roosts within each quartile

The average size of patches containing zero, one or multiple roosts was significantly different in the first aggregated quartile (i.e. the quartile of the smallest patches; Figure 3.4; Table 3.3); patches occupied by multiple roosts were larger than patches occupied by a single roost and both categories of occupied patches were larger than unoccupied patches. This was confirmed with a Games-Howell *post hoc* test (Table 3.4). There was no significant different between patches containing zero, one or multiple roosts within aggregated quartiles 2, 3 and 4 (Figure 3.4; Table 3.5).

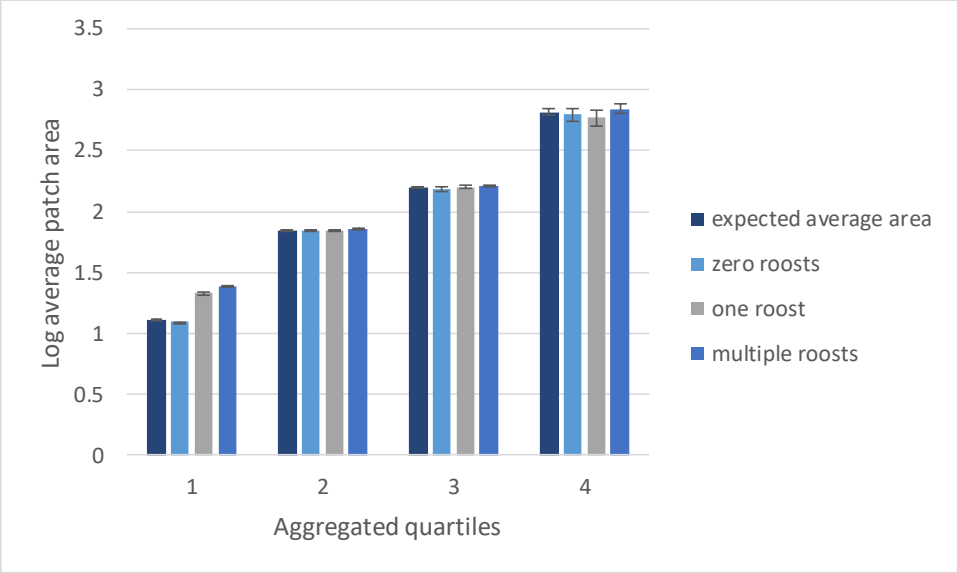


Figure 3.4 The average area (km^2, log scale) of road-defined patches containing zero, one or multiple roosts for each aggregated quartile as well as the expected average area of each category of patch.

					ANOVA					
		Zero roosts	**One roost**	**Multiple roosts**	**df effect**	**df error**	**MS**	**F**	**F crit**	**p-value**
Quartile 1	Mean area	3.36	13.5	17.67	2	12598	70082.23	1358.29	2.99	<0.001***
	Number of patches	11557	566	478						
	Number of roosts	0	566	1870						
Quartile 2	Mean area	61.73	61.92	64.44	2	870	728.49	2.88	3	0.05
	Number of patches	385	146	332						
	Number of roosts	0	146	1801						
Quartile 3	Mean area	152.38	155.52	162.05	2	344	3134.09	0.99	3.02	0.37
	Number of patches	91	64	202						
	Number of roosts	0	64	1339						
Quartile 4	Mean area	715.35	698.1	811.55	2	69	95999.2	0.32	3.12	0.72
	Number of patches	21	13	38						
	Number of roosts	0	13	373						

Table 3.3 The average area and number of road-defined patches containing zero, one or multiple roosts, the number of roosts in each type of patch and the results of ANOVAs.

							95% confidence interval			
group 1	group 2	Mean difference	std err	q-stat	df	q-crit	lower	upper	p-value	mean-crit
zero	one	10.13134	0.3315	30.56207	628.6432	3.314	9.032746	11.22993	<0.0001	1.098592
zero	multiple	14.30732	0.398793	35.87652	449.7304	3.325207	12.98125	15.63339	<0.0001	1.32607
one	multiple	4.175981	0.514889	8.110455	931.5387	3.314	2.46964	5.882322	<0.0001	1.706341

Table 3.4 The results of a Games Howell *post hoc* test confirmed a significant difference between the average size of patches containing zero and one roost, zero and multiple roosts and one and multiple roosts in the first aggregated quartile. Specifically, patches with one roost were larger than patches with zero roosts, and patches with multiple roosts and larger than patches with a single roost.

3.4.1.5 Patch quality

Minor road density was lower in the occupied than the unoccupied patches of the first area quartile, which was composed of the smallest patches (Figure 3.5 a; Table 3.5). In contrast, the density of minor roads was higher in occupied than unoccupied patches in the remaining three quartiles (Figure 3.5 a; Table 3.5).

Occupied patches consistently had a greater area of broadleaved woodland than unoccupied patches (Figure 3.5 b; Table 3.5) whereas occupied patches contained more coniferous woodland than unoccupied patches in the first quartile only (Figure 3.5 c; Table 3.5). Occupied patches had less coniferous woodland than unoccupied patches for the second and third quartiles (Figure 3.5 c; Table 3.5).

Occupied patches had a significantly greater area of grassland than unoccupied patches in the first quartile only (Figure 3.5 d; Table 3.5) and occupied patches had a greater area of built environments in all four quartiles (Figure 3.5 e; Table 3.5).

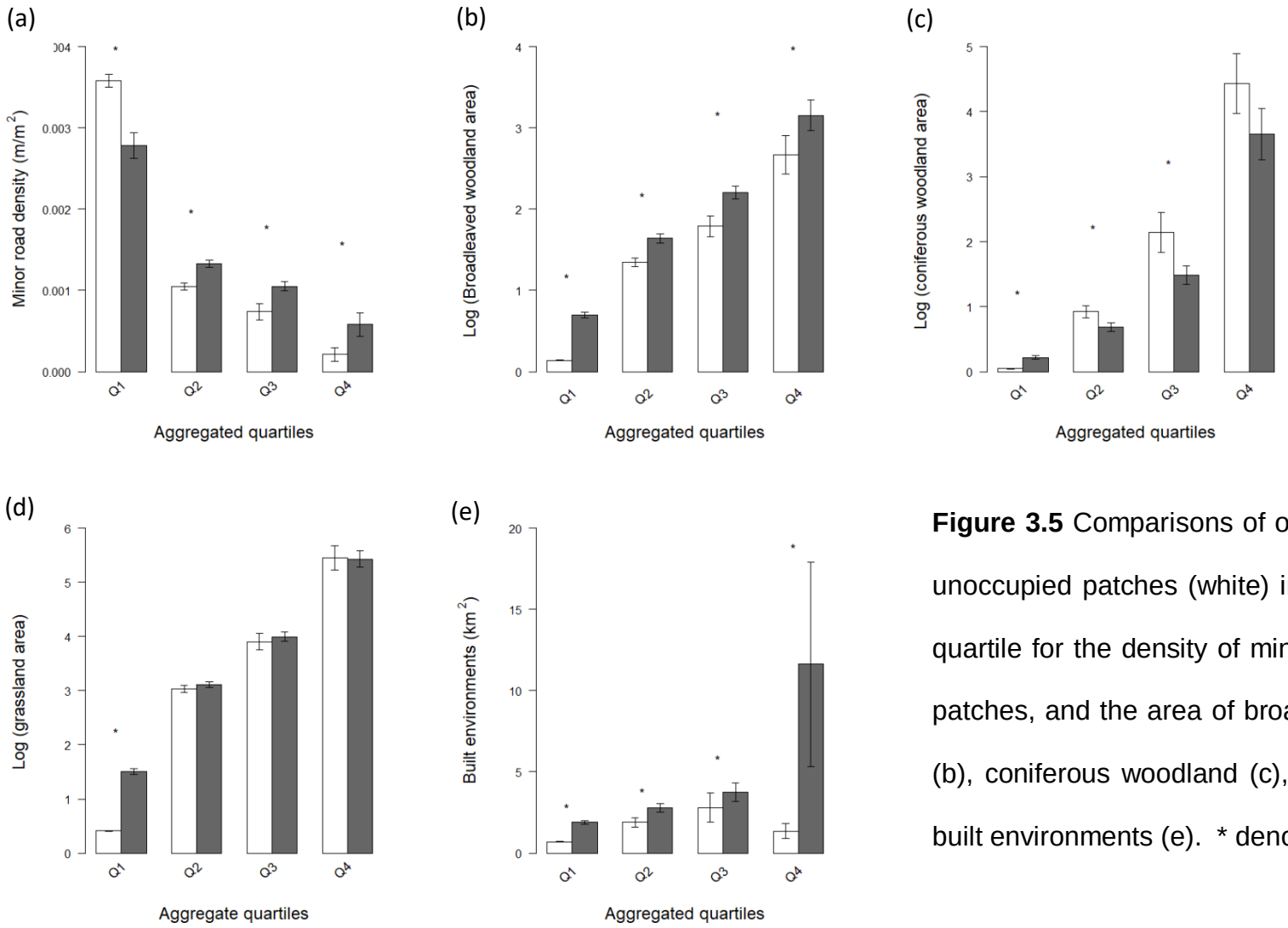


Figure 3.5 Comparisons of occupied (grey) and unoccupied patches (white) in each aggregated quartile for the density of minor roads (a) within patches, and the area of broadleaved woodland (b), coniferous woodland (c), grassland (d) and built environments (e). * denotes significance.

Comparisons between occupied and unoccupied patches					
		Mean values		Wilcoxon rank sum	
Figure 5		Unoccupied	Occupied	W	p-value
(a)	**Minor road density (m/m^2)**				
	Quartile 1	0.00357	0.00278	5657700	< 0.001***
	Quartile 2	0.00104	0.00133	62424	< 0.001***
	Quartile 3	0.00074	0.00105	7203	< 0.001***
	Quartile 4	2.14E-04	5.82E-04	296	< 0.01**
(b)	**Broadleaved woodland (km^2)**				
	Quartile 1	0.22	1.4	1762500	< 0.001***
	Quartile 2	3.43	5.27	67391	< 0.001***
	Quartile 3	6.03	10.13	7108	< 0.001***
	Quartile 4	15.68	28.18	306	< 0.01**
(c)	**Coniferous woodland (km^2)**				
	Quartile 1	0.08	0.42	2878400	< 0.001***
	Quartile 2	3.16	1.81	105120	< 0.01**
	Quartile 3	18.21	8.4	14164	< 0.001***
	Quartile 4	126.97	78.38	676	0.082
(d)	**Grass (km^2)**				
	Quartile 1	1.15	5.63	1780900	< 0.001***
	Quartile 2	24.2	24.85	89694	0.23
	Quartile 3	60.43	65.42	10512	0.32
	Quartile 4	266.67	262.54	540	0.96
(e)	**Built environment (km^2)**				
	Quartile 1	0.68	1.89	629700	< 0.001***
	Quartile 2	1.9	2.78	64002	< 0.001***

Quartile 3	2.79	3.74	8065	< 0.001***
Quartile 4	1.36	11.62	260	< 0.001***

Table 3.5 The mean values of occupied and unoccupied patches for each aggregated quartile for minor road density (Figure 3.5 a) broadleaved woodland (Figure 3.5 b), coniferous woodland (Figure 3.5 c), grassland (Figure 3.5 d), and built environments (Figure 3.5 e).

1257 **3.4.1.6 Regression analysis**

1258 The proportion of patches occupied by at least one roost increased with each
1259 incremental increase in patch size of 5 km^2 (Figure 3.6 a; $R^2 = 0.073$).

1260 The proportion of occupied patches decreased sharply as minor road density
1261 increased from 0 to 20 km/km^2 and remained at zero at higher densities (Figure 3.6 b;
1262 $R^2 = 0.95$).

1263 There was a non-linear decrease in the proportion of occupied patches with
1264 increasing percentage area of broadleaved woodland (Figure 3.6 c; $R^2 = 0.93$),
1265 coniferous woodland (Figure 3.6 d; $R^2 = 0.47$) and grassland (Figure 3.6 d; $R^2 = 0.88$).

1266 The proportion of occupied patches peaked at 20-25% broadleaved woodland, at
1267 approximately 40% for coniferous woodland and at 55-60% grassland.

1268 As the percentage of built environment within a patch increased, the proportion of
1269 occupied patches decreased (Figure 3.6 e; $R^2 = 0.94$).

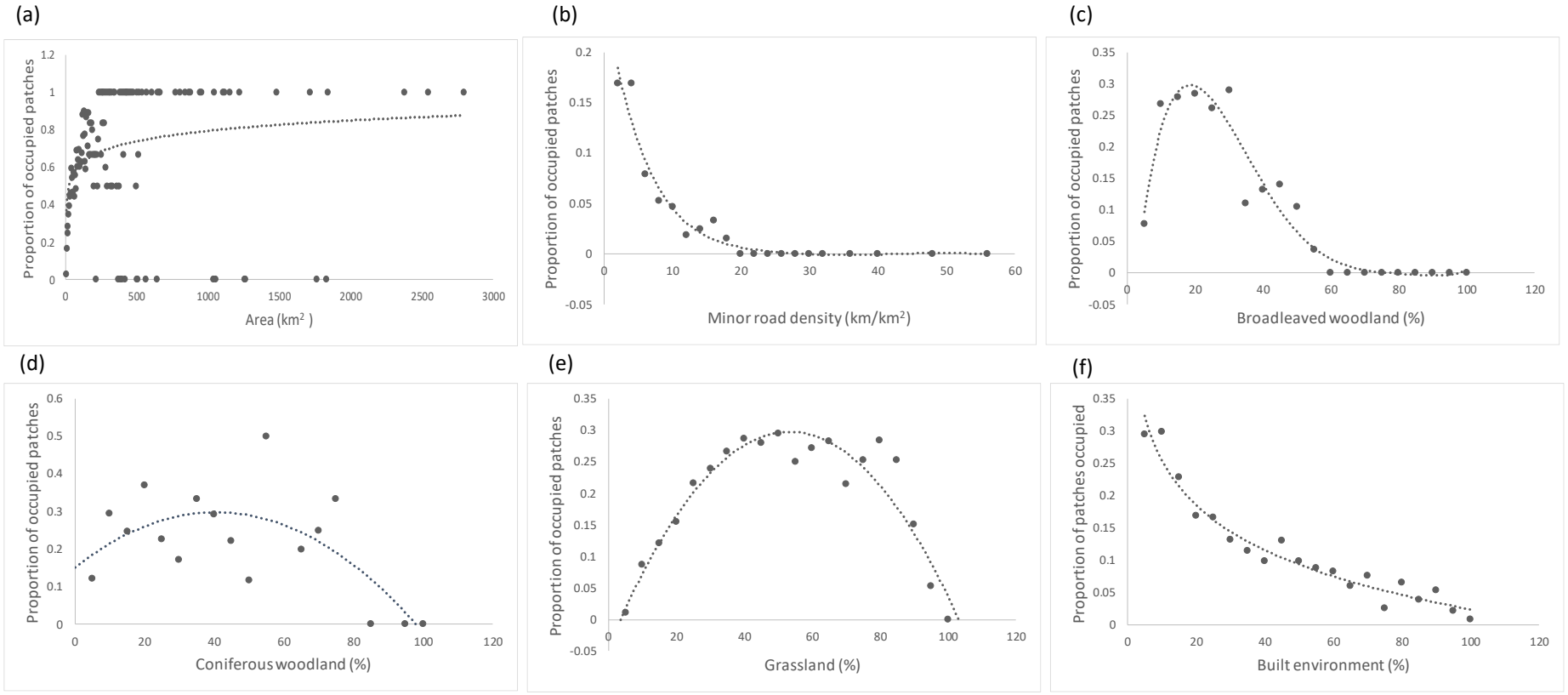


Figure 3.6 The proportion of occupied patches in each bin for patch size (a), minor road density (b), broadleaved woodland area (c), coniferous woodland area (d), grassland areas (e) and the area of built environment (f).

3.4.1.7 Multi-model inference and binomial generalized linear model

All variables selected with a relaxed p-value ($p < 0.1$) were included in the top model and most variables were included in all models (Table 3.6). Consistent with the pattern established by the analysis of area quartiles, patch size influenced the likelihood of roost presence within a patch; as patch size increases, the probability of a roost being present within a patch increases by 0.02%, with each additional square kilometre, as indicated by the odds ratio (1.02, Table 3.6). Minor road density within patches also had a positive influence on roost presence; the probability of a roost being present increases by 57% for each additional kilometre of road per square kilometre. For each percentage increase in the area of broadleaved woodland, coniferous woodland, grassland or built environment within a patch, the probability of a roost being present declined by between 2% and 5%.

The model allows us to make predictions with regards to the probability of a roost being present within a patch under different environmental scenarios (Figure 3.7-3.9). The probability of a roost being present within a patch of 50km^2 increases from 0.4 if the patch contains 1% broadleaved woodland, to a probability of 1 if the patch contains 100% woodland whilst all other variables are held at their means (Figure 3.7).

Model		AIC	ΔAIC	Akaike weight
1	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22	7038.32	0	0.58
2	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 19 20 21 22	7039.99	1.67	0.25
3	1 2 3 4 5 6 7 8 9 10 11 12 13 14 16 17 18 19 20 21 22	7040.78	2.45	0.17

Variable	Code	Predictor weight	Coefficients	Odds ratio	95% CI for multivariate odds ratio
Intercept			-1.32		
Patch size (km^2)	1	1	0.021	1.02	0.01 – 0.02
Broadleaved woodland (%)	2	1	-0.042	0.95	-0.06 – -0.02
Coniferous woodland (%)	3	1	-0.023	0.97	-0.06 – 0.01
Minor road density (km/km^2)	4	1	0.45	1.57	0.31 – 0.59
Grassland (%)	5	1	-0.018	0.98	-0.03 – -0.0048
Built environment (%)	6	1	-0.043	0.95	-0.05 – -0.03
x co-ordinate	7	1	-0.000002	0.99	-3.75E-06 – -5.05
y co-ordinate	8	1	-0.000001	0.99	-1.42E-06 – -5.97
Patch size: broadleaved woodland	9	1	0.001685	1.00	0.0012 – 0.0021
Patch size: Coniferous woodland	10	1	-0.0003112	0.99	-3.9E-04 – -2.28E-04
Patch size: minor road density	11	1	0.007881	1.00	0.0035 – 0.0122

Patch size: grassland	12	1	-0.0001452	0.99	-2.1E-04 – -7.10E-05
Patch size: built environment	13	1	0.001474	1.00	9.34E-04 – 0.002
Patch size: y co-ordinate	14	1	-1.779E-08	0.99	-2.52E-08 – -1.03E-08
Broadleaved woodland: grassland	16	1	0.0009518	1.00	5.01E-04 – 0.0014
Broadleaved woodland: built environment	17	1	0.0009021	1.00	4.67E-04 – 0.0013
Minor road density: grassland	19	1	-0.003535	0.99	-0.0054 – -0.0015
Minor road density: built environment	20	1	-0.003593	0.99	-0.0049 – -0.0021
Grassland: x co-ordinate	21	1	6.422E-08	1.00	3.27E-08 – 9.57E-08
Built area: x co-ordinate	22	1	3.541E-08	1.00	7.98E-09 – 6.28E-08
Broadleaved woodland: coniferous woodland	15	0.83	0.001105	1.00	9.83E-05 – 0.0021
Coniferous woodland: x co-ordinate	18	0.75	9.672E-08	1.00	-3.29E-09 – 1.96E-07

Table 3.6 The top three models obtained by multi-model inference as well as the odds ratios and confidence intervals for each variable in the top model.

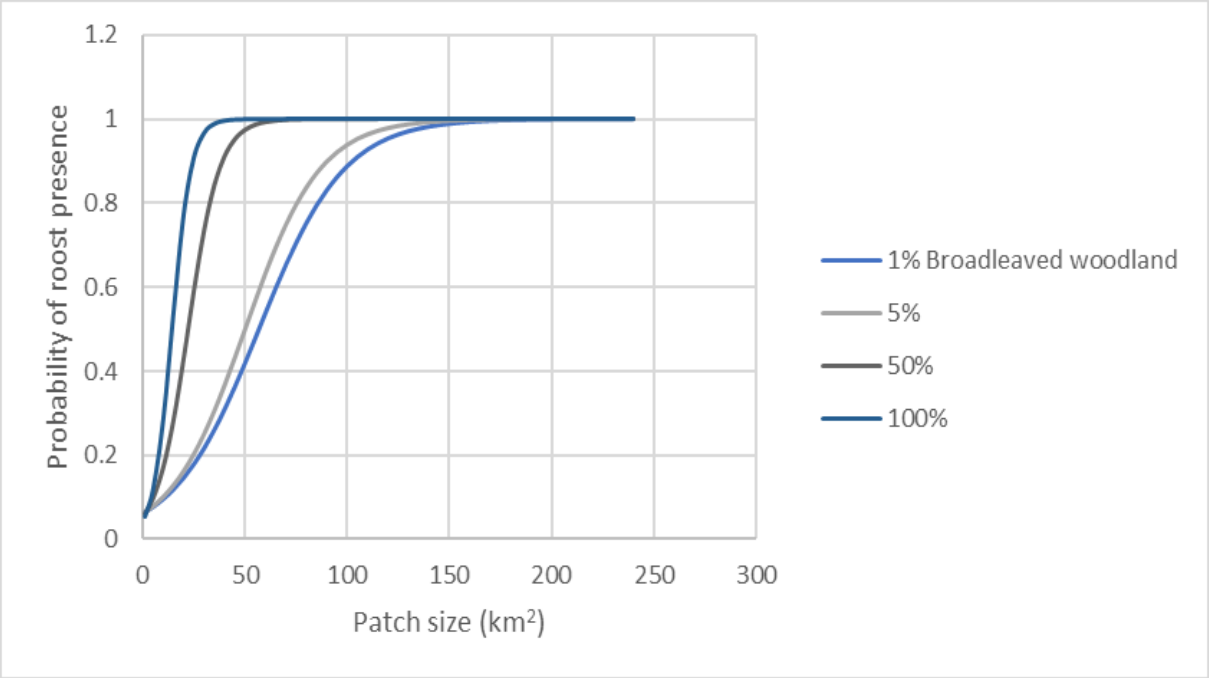


Figure 3.7 The probability of a roost being present within a road-defined patch of a given size, with 1, 5, 50 and 100% broadleaved woodland within the patch. All other variables are set at their mean values (coniferous woodland 1.5%; grassland 20%; minor road density 0.003 m/m^2) except for built environments which was set at 20% coverage within a patch rather than a mean of 50% for the sake of clarity. The geographical co-ordinates for the hypothetical patches are for a location in the South West of England.

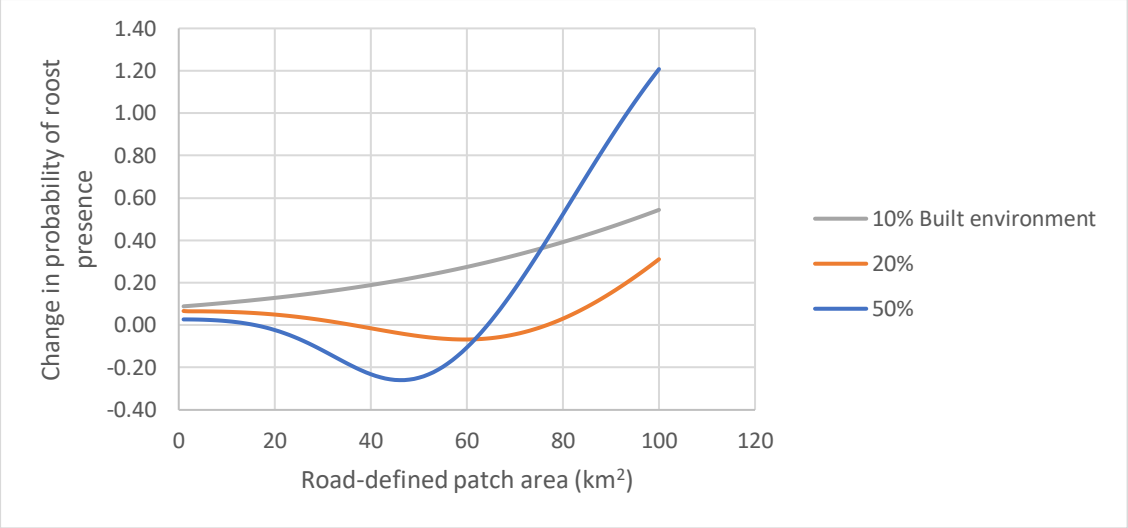


Figure 3.8 The change in the probability of roosts being present in two new equally sized road-defined patches following fragmentation of the orginal patch by a new road. I include estimates for patches with 10, 20 and 50% built environments. All other variables have been set at their mean (broadleaved woodland, 5%; coniferous woodland, 1.5%; grassland, 20%; minor road density, 0.003 m/m^2).

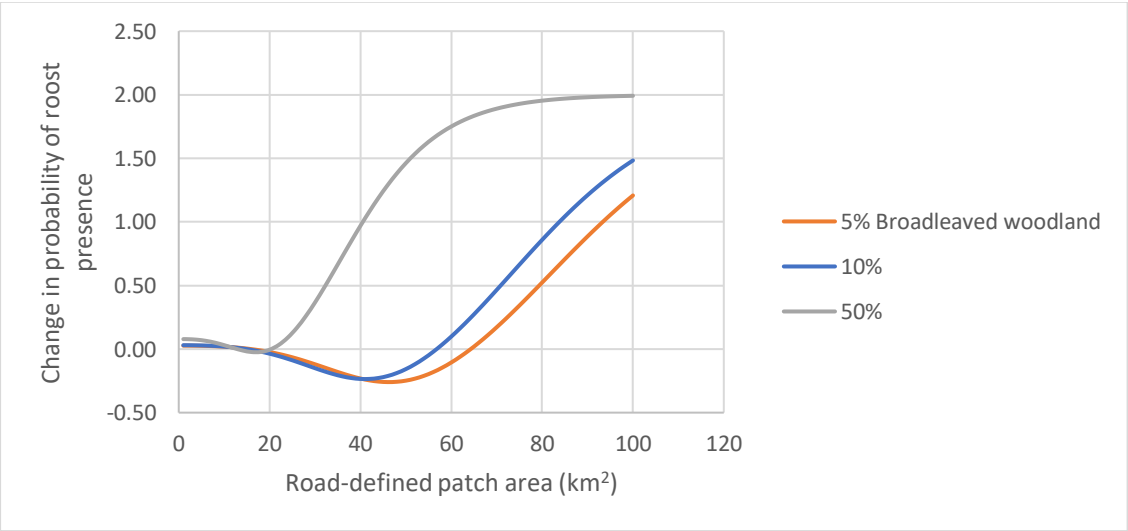


Figure 3.9 The change in the probability of roosts being present in two new equally sized road-defined patches following fragmentation of the orginal patch by a new road. I include estimates for patches with 5, 10 and 50% broadleaved woodland. All other

variables have been set at their mean (built environments 50%; coniferous woodland 1.5%; grassland 20%; minor road density 0.003 m/m^2).

3.4.1.8 Exploring robustness and generality

The analyses of individual species and the genera *Pipistrellus* and *Myotis* (Appendices 3.3-3.12*)*, and patches with 0-80%, 0-20% and 80-100% built environments (Appendices 3.13-3.22), produced results highly consistent with those of the primary analysis. In all cases, abundance of roosts declined across quartiles whilst the proportion of occupied patches increased. Measures of habitat quality showed the same overall trends in most analyses to those of the primary analysis, although the differences between occupied and unoccupied patches were not always significant.

There were a few notable exceptions. First, in the dataset containing patches with 80-100% built environments, the abundance of roosts within a quartile increased in the fourth quartile compared to the second and third (Appendix 3.13). Second, in the dataset containing patches with 80-100% built environments, the tendency for roost prevalence to increase across quartiles was much more pronounced (Appendix 3.14). Finally, in the datasets containing patches with 0-20% and 80-100% built environments, occupied patches had a greater density of minor roads than unoccupied patches in the first quartile (Appendix 3.15) in contrast to the results from the complete dataset and the subset data with patches containing 0-80% built environment which both showed a lower density of minor roads in the first quartile (Appendix 3.15).

In summary, the results presented in the main analysis are robust and generalisable across species or genera and in areas with very high levels of

urbanisation (80-100% built environments), very low levels of urbanisation (0-20% built environments) and where all but the most urbanised patches are considered (0-80% built environments). This suggests that the road-defined patch size effect in the main analysis does not simply reflect avoidance of highly urbanised areas.

3.5 Discussion

I present strong evidence that the distribution of bat roosts in the UK is related to fragmentation of the landscape by roads. The likelihood of a roost being present within a patch is area-dependent and the total area occupied by roosts within an aggregated quartile increased as the degree of fragmentation decreased.

While the abundance of roosts was greatest in the quartile composed of the smallest patches, prevalence (relative frequency of occupied patches) was lower, i.e. these roosts are concentrated in a much smaller area. This occupied area was composed of patches that were, on average, larger with a lower density of minor roads and a greater area of coniferous and broadleaved woodland and grassland than patches in the unoccupied area.

Results were very similar across species/genera, suggesting a high degree of generality. Area-dependent patch occupation was demonstrated even by *Eptesicus serotinus* and the genus *Pipistrellus* which are thought to be more likely to traverse roads and therefore less likely to be constrained by patch size.

Analysis of the least urbanised patches (0-20% built environments), the most urbanised patches (80-100% built environments) and of patches after having excluded the most highly urbanised patches (0-80% build environments) also produced results

consistent with the primary analysis. This suggests that area-dependent patch occupation does not reflect, or solely reflect avoidance of small, urbanised patches but that preference for larger patches also exists in the wider countryside.

3.5.1 Predicting the impact of new roads, or mitigation, on bat roosts

The model presented here allows us to predict the effect of removing roads as an impediment to landscape scale movement and improving habitat quality on roost presence. For example, a road-defined patch of 10 km^2 (where all other environmental variables are at their mean; broadleaved woodland 5%; built environments 50%; coniferous woodland 1.5%; grassland 20%; minor road density 0.003 m/m^2) has a 0.1 probability of containing a roost. By joining five similar sized patches, perhaps by building green bridges, the probability of a roost being present within what is now a 50 km^2 patch increases to 0.49. By replanting trees so that broadleaved woodland coverage increases from 5% to 16% the probability of a roost being present increases to 0.7.

Conversely, we can predict the impact of bisecting an existing road-defined patch. If a road-defined patch of 50 km^2 is bisected by a new road, and all other environmental variables are held at their mean, the probability of a roost being present somewhere in the newly fragmented area declines from 0.49 to 0.44. If this area, in addition to being fragmented by a new road, were to suffer deforestation so that broadleaved woodland coverage fell from a mean of 5% to 1%, the probability drops further to 0.39.

3.5.2 Implications for the conservation management of bats

3.5.3 Preserve

Our study indicates that further fragmentation by roads could impair the persistence of roosts within patches. The construction of new major roads could reduce the probability of roost presence by reducing the size of a patch. Given that the majority of patches defined by major roads were smaller than 3km^2, the smallest core sustenance zone required by bat species in the UK, it is essential that the size of patches be preserved, especially the remaining patches that are greater than 3km^2. Additional minor roads within the smallest patches could reduce patch quality.

3.5.4 Connect

As well as preserving the size of existing patches, it is essential that connectivity between patches be increased in order to maximise the resources available to local bat populations and to encourage the recolonization of patches. Connectivity at the landscape scale is also important in the context of range shifts in response to climate change (Schloss et al. 2012; Lawton et al. 2010). At the same time, it is important to consider that encouraging bats to cross roads could increase the risk of collision with traffic (Fensome & Mathews 2016). Bat bridges have been shown to be an ineffective means of connecting habitats bisected by roads; most bats do not cross at the gantry and those that do, do so at an unsafe height (Berthinussen & Altringham 2012b). Bridges, including green bridges, could be a safer and more effective aid to landscape scale movement for bats and a range of other species (Natural England 2015; Bhardwaj et al. 2017).

3.5.5 Improve

Improving the quality of patches by restoring broadleaved woodland could also increase the likelihood of roost persistence and re-colonisation of smaller patches. Woodlands are a key habitat for many species of bat in the UK. Broadleaved woodlands may be a more useful habitat than coniferous woodlands as coniferous woodlands are thought to offer fewer opportunities for foraging and roosting (but see Kirkpatrick et al. 2017). Bat activity levels have been shown to be higher in broadleaved than coniferous woodlands (Walsh & Harris 1996) and in another study, the location of bat roosts for six species were associated more strongly with the proximity and extent of broadleaved woodland than coniferous woodland (Boughey et al. 2011). Our results are consistent with these studies' findings. However, our results also suggest that coniferous woodlands may provide valuable foraging opportunities in the smallest patches.

3.5.6 Future research

It is a weakness of the present study that cartological ecology establishes only correlations and does not by itself establish their causal basis. Future research should aim to establish the mechanistic basis of the correlations established by the present study.

There are potentially two primary mechanisms that could explain the apparent preference for larger patches for roosting by bats. The first is that roads, by acting as a barrier to movement, largely limit access to resources within the patch. The smaller the patch, the fewer the resources. Consequently, smaller patches may be less able to support sustainable populations.

The second mechanism is related to road-related mortality. Animals living in smaller or poorer quality patches may be more likely to travel to adjacent patches to access additional resources. Regularly commuting to adjacent patches could increase the frequency with which animals encounter traffic and increase the likelihood of collision with vehicles. Consequently, populations living in smaller patches could suffer unsustainable additional mortality; i.e. there may exist patch size-dependent road-related mortality.

In order to develop a better understanding of the potential underlying mechanisms for road-defined patch selection, the next steps are to investigate the influence of patch size and quality on: 1. average roost size and changes in roost size over time, which can be calculated from the NBMP roost visit records; and 2. the probability of road crossing and collision rates whilst controlling for patch permeability. Permeability is likely to be determined by boundary attributes such as road width, traffic density, lighting regime and the presence and proximity of bordering vegetation. Attributes of the focal species will also determine the permeability of a boundary, such as their behavioural response to roads, habitat size requirements, and risk of collision with vehicles.

3.5.7 Differences between roost types

Future research should also examine whether there are differences between different types of roost in their response to fragmentation by roads. The practical implication of this research is to identify which types of roost are more vulnerable to the construction of new roads and whether the same level of protection should be

afforded to all roosts, including all-male roosts, mixed day and hibernation roosts, or whether the protection of maternity roosts should be prioritised.

We might expect that maternity roosts are much more likely to be located in larger patches than all-male or mixed day roosts and to be more sensitive to further fragmentation by roads. There is growing evidence that, at least for some species, the sexes segregate according to habitat quality with females dominating optimal and less degraded habitats (Senior et al. 2005; Mackie & Racey 2007; Angell et al. 2013; Lintott et al. 2014). The greater energetic requirements of lactating and pregnant females could mean that females in maternity roosts are more sensitive to patch size and quality than males and non-breeding females in day and hibernation roosts.

3.5.8 Beyond bats

The analyses conducted here, applied to other taxa, could establish the basis for a direct comparison of the vulnerability to habitat fragmentation by roads among a broad range of species. Specifically, it may be possible to directly compare the coefficients of patch size or habitat quality on the probability of presence within a patch, obtained from GLM models.

By comparing the coefficients from multiple species, it may also be possible to identify ecological or behavioural traits that are strongly associated with greater vulnerability to road-defined patch size or patch quality such as behavioural responses to roads, traffic collision rates and range requirements.

Furthermore, knowledge of the impact of road-defined patch size on multiple species would allow us to to make predictions about the impact of a new road on a community of species rather than a single species, which could inform planning

decisions with regards to new roads. Specifically, it may be possible to identify the route that has the least ecological impact by calculating the predicted impact of a new road bisecting multiple patches of varying sizes, on the presence of several species in the newly fragmented areas. For each species in each original patch, it would be possible to calculate the change in the probability of species presence between the original road-defined patch (or unfragmented area), and the combined probability of species presence in the area now divided into multiple patches. Combining these changes in the probability of presence for multiple species following the construction of a new road, provides an overall “impact score” for a particular route.

It may also be possible to make predictions about the downstream effects of roads on predators by examining the coefficients of prey species. For example, it may be the case that insufficient data exists to model the impact of road-defined patch size on certain, rare or less well monitored predators such as birds of prey. But it may be possible to predict the impact of new roads on well-documented prey species such as small mammals, and to extrapolate from that whether a new road is likely to have an effect on the availability of a predator’s food source.

Future analyses could also be extended to correlate patch size with species diversity and abundance.

3.5.9 Wider implications and conclusions

The road network can usefully be seen as casting a net across the landscape that acts as a filter through which some species, and the ecological processes they are a part of, are better able to pass than others. By acting as a barrier to movement, roads could create ecological islands with species assemblages and abundances

determined by the size of patches and the degree to which patches are isolated from each other by bordering roads.

The results presented here emphasise the importance of management at the landscape scale for the conservation of biodiversity. Our proposal that road-defined patches in the UK ought to be preserved, connected and improved echo those of Lawton *et al.* who called for protected areas in England to be "*bigger*, *better* and *joined".* In "making space for nature", Lawton and colleagues made the case for landscape scale conservation in the context of protected areas in England (Lawton et al. 2010) and recommended that they be increased in size, improved through better habitat management and connected by corridors and habitat "stepping-stones". However, roads could be a serious impediment to a well-connected ecological network.

Further investigation is needed to ensure that protected areas function as ecologically continuous areas and that roads within protected areas, as elsewhere, are traversable without increasing the risk of collisions with wildlife.

3.5.10 Comments and caveats

3.5.11 Replicate records of roosts

There are multiple records for some roosts; i.e. there are records with the same co-ordinates and species identity. In itself, this is unlikely to have strongly affected the strongest patters established by our analyses. Nevertheless, it bears some discussion as follows. There are two explanations. First, it is possible that Natural England and the BCT hold records of the same roosts. Second, these are not replicates at all but represent separate roosts of the same species in very close proximity. The location

data provided within the dataset may not have been sufficiently accurate to distinguish between these very close roosts.

The number of potentially erroneous replicates was highest amongst patches in the first aggregated quartile (Figure 3.10). As quartiles increased the number of replicates decreased. As most of the smaller patches were in England, this could indicate that the replicates were indeed roosts reported by both BCT and Natural England.

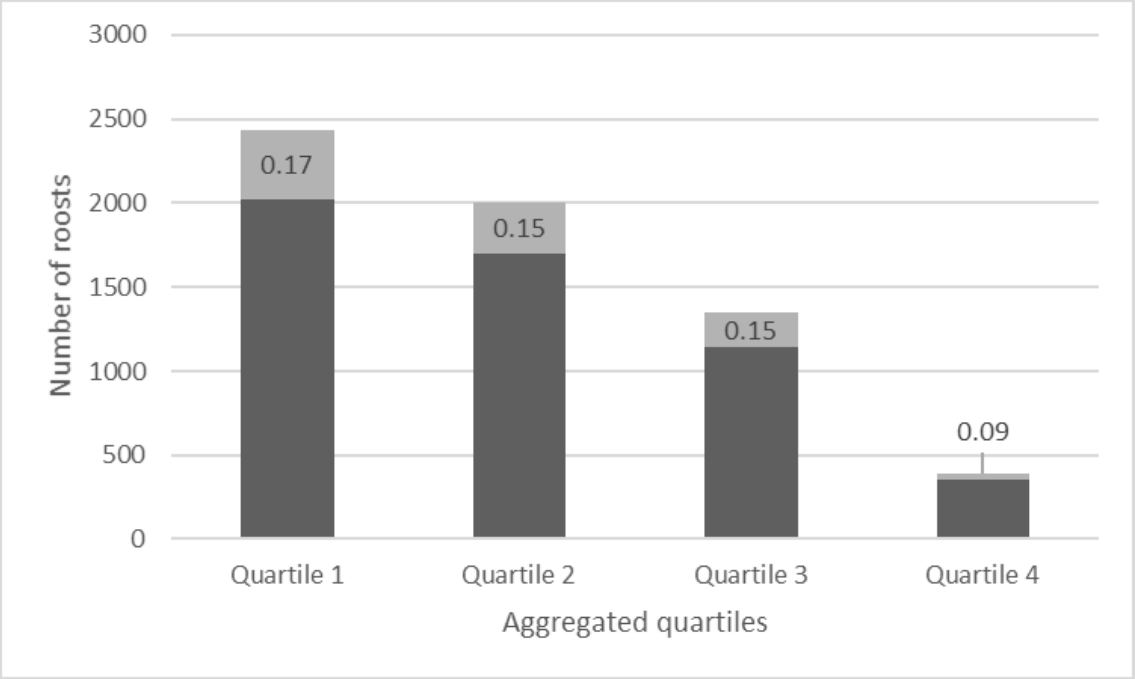


Figure 3.10 The number of potentially erroneous replicates (light grey) out of all roosts in each quartile (dark and light grey). The proportion of all roosts that these potential replicates represent are also provided.

These replicates have no effect on patch occupancy (prevalence) analyses but could affect the roost number (abundance) analyses. For this reason, I corrected the

dataset and repeated the analysis of roost abundance in each aggregated quartile, with all species included. However, this had no effect on the overall results. After having removed replicates, there were still more roosts in the first aggregated quartile and the number declined for each subsequent aggregated quartile (Figure 3.11 b; Pearson's chi-squared test, χ^2= 1232.51, df=3, p<0.001).

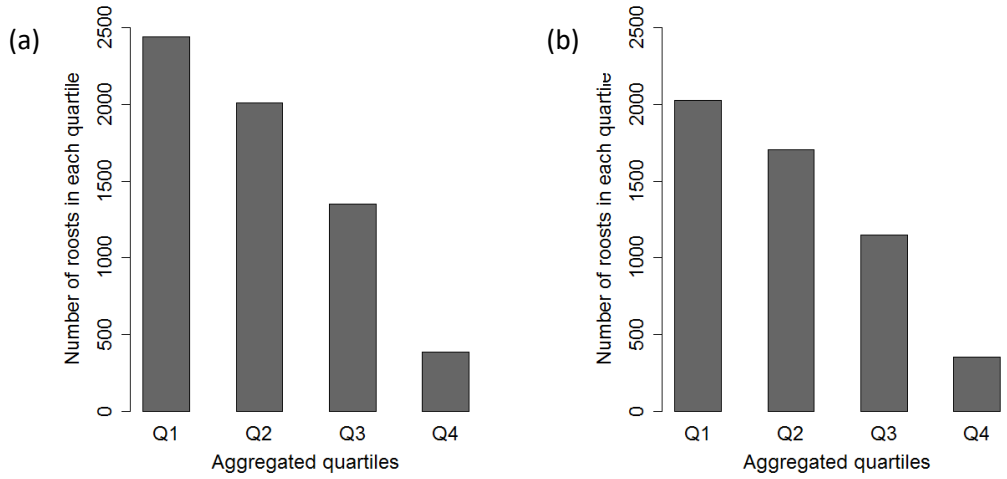


Figure 3.11 The number of roosts in each quartile for the original analysis, including potentially erroneous replicates (a; n=6192), and without (b; n=5226). The total number of patches in each analysis, n=13893.

3.5.12 Confounding geographic influences on roost distributions

In the original analysis, most of the patches in the first aggregated quartile, containing the smallest patches, were in England whereas most of the patches in the fourth quartile, composed of the largest patches, were in Scotland. There are more roosts in England (original dataset n=5606; without replicates n= 4633) than Scotland (original dataset n=273; without replicates n=273). There are two potential reasons why there are more records of roosts in England than Scotland. First, I supplemented

records from the BCT's NBMP dataset with records from Natural England but did not have access to an equivalent dataset for Scotland. Second, the distributions of some species (e.g. *Rhinolophus hipposideros, R. ferrumequinum, Myotis bechsteinii*) are confined to southern England. Other species are sparsely distributed at the northern limits of their range (e.g. *Myotis nattereri*, *M. daubentonii*, *Pipistrellus pygmaeus*).

In order to examine whether the results from the original analysis were an artefact of the distribution of some species, I repeated the analysis of roost abundance in aggregated quartiles for England (Figure 3.12 a) and Scotland (Figure 3.12 b) separately, after having removed replicates. However, the results were similar to the original analysis; abundance was greatest in the first aggregated quartile and declined thereafter (Pearson's chi-squared test; England, χ^2=275.01, P<0.001; Scotland, χ^2=124.67, P<0.001). Consequently, I conclude that the general patterns that I established are likely to be robust – i.e. that roads are influential in both major regions of the UK.

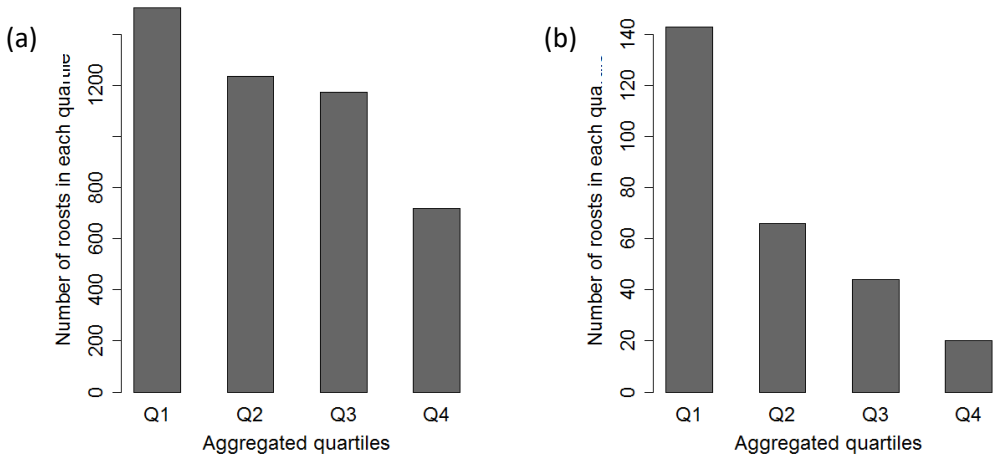


Figure 3.12 The number of roosts in each aggregated quartile in England (a; n=4633) and Scotland (b; n=273). Roads create 11,144 patches in England and each

aggregated quartile is 32,692 ± 51 km^2. Roads create 1831 patches in Scotland and each aggregated quartile is 17,134 ± 261 km^2.

3.5.13 Sampling bias

Some of our results may be affected by sampling bias. But I argue that the effects are likely to be conservative. Sampling bias likely exists in the datasets from which I obtained roost locations. The largest road-defined patches tend to have a lower density of minor roads and a lower proportion of built environments, and are by definition, remote. They also have the greatest proportion of woodlands. It is possible that roosts in patches with these qualities are less likely to be encountered by people.

This would mean that roost abundance and prevalence in larger patches may have been underestimated, and could also explain why the proportion of occupied patches in the fourth quartile that consists of the largest patches is slightly lower than the third quartile. The apparent preference for patches with a greater density of minor roads in the largest three quartiles (Figure 3.4 a) and the absence of roosts in patches containing more than 60% broadleaved woodland (Figure 3.5 c), may also, be an artefact of this sampling bias.

The most significant cause for concern is the influence of sampling bias on model predictions. As Figures 3.8 and 3.9 show, the predicted impact of new roads on bisecting very large patches (in the region of 60km^2 and above) is to increase the likelihood of a roost being present in the remaining, smaller patches. I would argue that this could in part be an indication that roosts are more likely to be *encountered* when road-defined patches are smaller.

3.5.14 Concluding remarks

Overall, this study begins to establish a framework for evaluating the threat to bats posed by the expanding road network and to map out a protocol useful in assessing risks in other animal species. Three major findings from this study are:

1. Roads influence the distribution of bat roosts by acting as an impermeable or partially permeable barrier to the movement of bats.

2. The probability of a roost being present within a patch is also related to the quality of the patch, namely, the proportion of broadleaved woodland accessible without crossing a road. Patch quality is especially important amongst the smallest patches. In the most fragmented hypothetical landscape (quartile 1), roosts were located in road-defined patches that were significantly larger, with lower minor road density and greater coverage by broadleaved woodland.

3. The model allows us to make predictions about the potential impact of a new road, or conversely the impact of linking patches and improving the quality of the habitat within them, on the probability of roost presence in the area of interest.

Chapter 4: An endocrinological investigation of the impacts of roads on bats

Thesis pp 168 - 290

4.0 Chapter 4: An endocrinological investigation of the impacts of roads on bats

4.1 Summary

Assessing the endocrinological state of free-living animals is an emerging method of investigating the impact of anthropogenic disturbance, which could potentially identify 'at risk' species and thereby inform conservation efforts. One group of hormones that are often the focus of such studies are the corticosteroids and their metabolites. At heightened levels, corticosteroids (specifically the glucocorticoids) mediate the stress response. At moderate levels they play a role in regulating energy, rising and falling in cycles (from daily to annual) in response to changes in energetic demands on the individual, such as arousal from sleep, foraging, breeding and hibernation (Landys et al. 2006; Romero 2002). Anthropogenic disturbance that reduces an organism's ability to meet its energetic demands or that causes an emergency stress response could affect glucocorticoid levels, which makes them a potentially useful bioassay.

Here, I investigate whether endocrinological assays are feasible and informative in faecal samples collected from bat roosts and use them to evaluate the presence of nearby roads. Specifically, I used a correlative approach to test whether variation in faecal corticosteroids and gonadal steroids were associated with the density of minor roads within the core sustenance zone (CSZ) and the proximity of roosts to nearby roads. I also used an experimental approach to test whether levels of faecal corticosteroids were affected by simulated street-lighting. Additionally, I

evaluated the effectiveness of my assays in discriminating among species and sexes of bats in order to validate the overall approach.

I found that the levels of corticosteroids and gonadal steroids quantified from faecal samples varied consistently among species and sexes of bats, which suggests that baseline levels may exist in unperturbed individuals. In the correlative investigation, the levels of corticosteroids increased with road density, which may be indicative of stress due to the detrimental impacts of roads on habitat quality and foraging success. At the same time, gonadal steroids decreased as corticosteroids increased which is typical of elevated stress levels.

In the experimental investigation, I found no detectable effect of artificial lighting on faecal steroids.

Overall, my results begin to establish faecal endocrinology as a useful and informative approach in assessing the impact of roads collectively on individuals within roosts and for thereby targeting conservation efforts.

4.2 Introduction

Hormone analysis is an emerging method of ecological endocrinology that can be used to assess an organism's or population's response to anthropogenic environmental change and disturbances (Bradshaw 2007; Busch & Hayward 2009; Ellis et al. 2012; Wikelski & Cooke 2006). One group of hormones that are commonly assessed are glucocorticoids and their metabolites (Busch & Hayward 2009; Sheriff et al. 2011).

Glucocorticoids mediate the stress response, including the initiation of an emergency life history stage (ELHS), and their presence or elevation can trigger a cascade of physiological and behavioural responses that enable the organism to cope and react appropriately to physical and psychological stimuli (Sheriff et al. 2011; Busch & Hayward 2009).

Whilst the stress response is adaptive, long-term exposure to glucocorticoids can have a range of deleterious impacts on individual organisms, which may in turn impact fundamental demographic rates (i.e. fecundity) and, hence, the population itself (Busch & Hayward 2009; Sheriff et al. 2011; Wikelski & Cooke 2006). If conservation researchers are able to link upstream measures (glucocorticoids) with downstream (population level) effects by appropriately quantifying the stress response in individuals, it may be possible to feasibly and conveniently identify a perturbation and begin actions to ameliorate the situation before effects are expressed at the level of the population, which may eventually cause population declines (Wikelski & Cooke 2006). Just as important from a conservation perspective is whether anthropogenic environmental change or disturbances influence glucocorticoids without eliciting a stress response, i.e. to what extent an organism or population can cope with environmental perturbations. Overall, therefore, ecological endocrinology may elucidate both stress and resilience in response to anthropogenic impacts.

In this chapter, I use a technical of faecal endocrinology, faecal corticosteroid and gonadal steroid analysis, to investigate the potential impact of roads on bats in the UK.

I begin this chapter by reviewing our knowledge of the physiological actions and behavioural changes mediated by glucocorticoids including its potential influence on

gonadal steroids (4.3). One of the key actions of glucocorticoids during the stress response is to support the individual's response to sudden increased energetic demands required to cope with a crisis. In fact, glucocorticoids constantly regulate energetic demands; there is a minimum (basal) requirement for glucocorticoids to regulate glucose, salt and water, and glucocorticoids also rise and fall according to predictable changes in energy requirements diurnally, seasonally and at different life history stages (McEwen & Wingfield 2003). Therefore, glucocorticoids operate (and vary) within three systems; basal, modulated and stress-related.

In section 4.4, I introduce the conceptual framework and justification for using faecal endocrinological analysis to investigate the impact of anthropogenic environmental change and disturbances on wild animals. In particular, I draw on McEwen and Wingfield's (2003) concept of 'allostasis', which is a unified conceptual model that integrates the functional significance of basal, modulated and stress-induced glucocorticoid levels and defines the stress response as the relationship between energy requirements and energy availability, allowing us to better understand how basal and modulated glucocorticoid levels mediate an organism's response to additional noxious stimuli, disturbances or environmental perturbations. With the aid of McEwen and Wingfield's conceptual framework, I formulate hypotheses about how roads could potentially influence glucocorticoid levels in bats through several pathways; for example, by reducing the availability of resources, increasing the energy invested in accessing resources as well as stimulating the stress response as a result of psychological stimuli such as vehicular traffic or artificial lighting.

In section 4.5, I present the results of a correlative study of faecal corticosteroid levels within maternity roosts against several variables related directly and indirectly to the presence of roads in core sustenance zones including road density, proximity

to roads and the availability of broadleaved woodland. I also report the outcome of an experimental study that I conducted with artificial lighting to directly examine one potential way in which roads could elicit a stress response in bats.

In order to use glucocorticoids as a means of measuring responses to conservation relevant variables, it is important to understand the many underlying sources of variation that exist and are consequently expressed as differences between and within individuals, sexes and species. I therefore conducted a comparative study to investigate variation amongst individuals and differences between sexes and species in order to validate methodological decisions made with regard to the correlative and experimental studies.

4.3 A review of the actions of glucocorticoids on the physiology and behaviour of animals

4.3.1 The HPA axis and glucocorticoids: the endocrinal signatures of stress

Below, I provide an overview of how glucocorticoids contribute to the stress response through the Hypothalamic-pituitary-adrenal (HPA) axis in Figure 4.1. Parallel and complimentary to this pathway (not shown), is the sympathetic nervous system (SNS). Activation of the SNS in response to a detectable stressor is almost instantaneous and is mediated by norepinephrine, epinephrine (also known as adrenaline) and catecholamine hormones which serve to increase arousal, elevate

heart rate and promote glycogenesis and lipolysis (Reeder & Kramer 2005; Romero 2004; Sheriff et al. 2011). For the purposes of this review, I will focus on the HPA axis.

The HPA pathway is in part mediated by glucocorticoids, i.e. cortisol and/or corticosterone. The predominant glucocorticoid will depend on the species; for fish and most mammals the predominant glucocorticoid is cortisol whereas in birds, rodents, amphibians and reptiles the predominant glucocorticoid is corticosterone (Romero 2004).

Glucocorticoids are steroid hormones that promote the conversion of protein and lipids to carbohydrates (Busch & Hayward 2009). I describe the impacts of glucocorticoids on the physiology and behaviour of organisms during a stress response in more detail in section 4.3.2.

At rest

Under normal conditions, the hippocampus inhibits the HPA axis. As glucocorticoid levels increase the hormone itself acts as a negative feedback signal (Reeder & Kramer 2005; Romero 2004).

Stimulation of the HPA axis

In response to a stressor (physical or psychological) the paraventricular nucleus (PVN) is stimulated to produce corticotrophin-releasing hormone (CRH). CRH then stimulates the pituitary to release adrenocorticotrophic hormone (ACTH; Reeder & Kramer 2005). ACTH then acts on the adrenal cortex which releases glucocorticoids (blue triangles). The quantity of glucocorticoids released depends on the severity of the stressor (Reeder & Kramer 2005).

Modulation by receptors

There are two types of receptor in the brain and body that mediate the actions of glucocorticoids and corticosteroids; mineralocorticoid receptors (also known as type I; green triangles) and glucocorticoid receptors (also known as type II; red triangles; Romero 2004; Sheriff et al. 2011). At low levels, glucocorticoids bind preferentially with high-affinity mineralocorticoid receptors. At high levels, glucocorticoids bind with low-affinity glucocorticoid receptors. Because glucocorticoids preferentially bind with mineralocorticoids, binding with low-affinity receptors largely occurs after mineralocorticoid receptors have been saturated (Reeder & Kramer 2005). This partly explains why glucocorticoids have different effects on the physiology and behaviour of organisms at low and high levels; at high levels glucocorticoids trigger an emergency life history stage (ELHS; Busch & Hayward 2009; McEwen & Wingfield 2003), the suite of rapid physiological and behavioural changes that help an organism overcome the immediate threat, described more fully below in section 4.3.2.

Cessation of the pathway

Cessation of glucocorticoid release is under the influence of glucocorticoids themselves through a negative feedback loop/system (Romero 2004). High concentrations interact with receptors in the hippocampus, hypothalamus and pituitary to suppress the initial steps of the HPA axis (Busch & Hayward 2009; Sheriff et al. 2011). In the event of an acute stress response, and the source of stress being overcome, glucocorticoid concentrations decline to basal levels within 60-90 minutes by metabolic elimination of the glucocorticoid (Sheriff et al. 2011). However, if the stressor is chronic, this feedback mechanism is weak and the entire system remains active for longer periods (Sheriff et al. 2011).

Metabolism and excretion

Glucocorticoids are destructively metabolised in the liver and metabolites and some parent compound are excreted via urine and faeces (Goyman 2013). Metabolites can be reabsorbed from the gut (Goyman 2013; Sheriff et al. 2011). The more time that waste remains in the gut, the more glucocorticoids are reabsorbed. Microbes in the gut can also break down metabolites further (Goyman 2013). Again, the more time glucocorticoid metabolites spend in gut, the more time microbes have to break down metabolites (Sheriff et al. 2011).

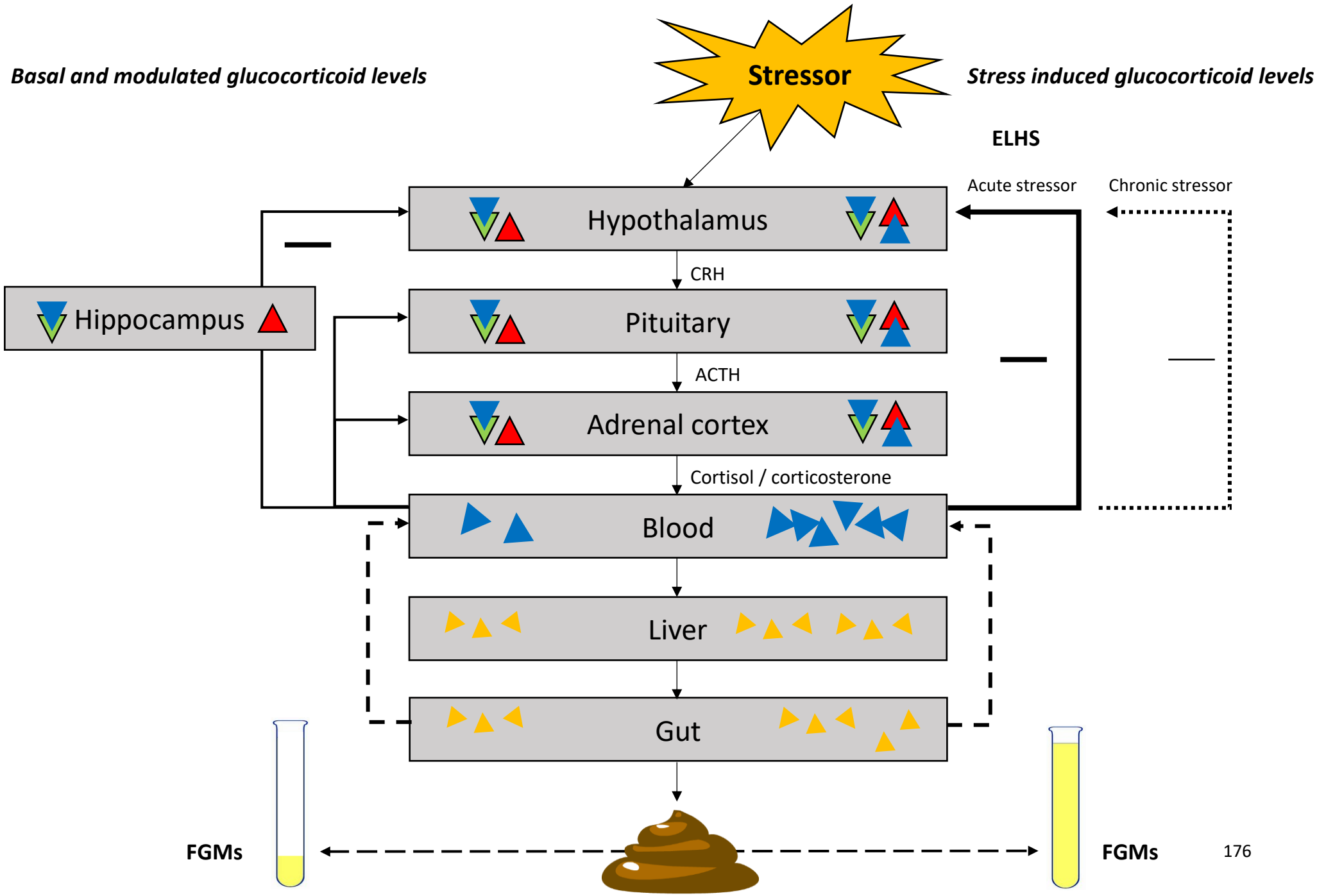

Basal and modulated glucocorticoid levels
Stressor
Stress induced glucocorticoid levels
ELHS
Acute stressor
Chronic stressor
Hypothalamus
CRH
Hippocampus
Pituitary
ACTH
Adrenal cortex
Cortisol / corticosterone
Blood
Liver
Gut
FGMs
FGMs

Figure 4.1 The HPA axis and the pathway of glucocorticoids at basal, modulated and stress induced levels.

4.3.2 The physiological and behavioural changes mediated by glucocorticoids

In order to appreciate the significance of variation in corticosteroid levels, it is necessary to have an understanding of the role of glucocorticoids in the HPA axis, which demonstrates that glucocorticoids operate within three homeostatic systems that map onto each other; basal, modulated and stress induced (Figure 4.2). The physiological and behavioural effects of glucocorticoids within these three systems depend upon the level and duration of glucocorticoid presence within the organism and are mediated in part by two receptors (as described above).

Fundamental to the effect that glucocorticoids have on physiology and behaviour at all levels is their role in regulating food intake and mobilizing existing energy stores. Many of the effects of glucocorticoids occur in conjunction with other hormones, such as epinephrine and insulin.

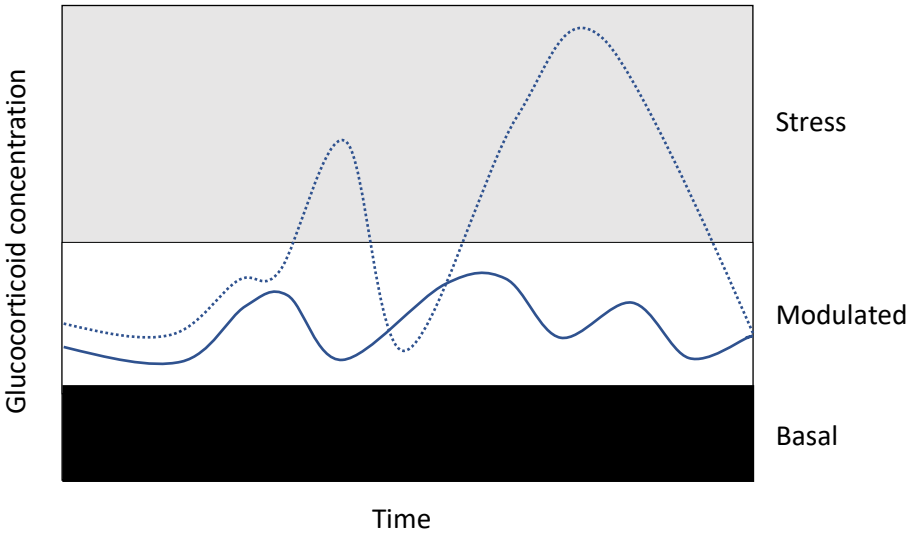


Figure 4.2 Diagram demonstrating how glucocorticoids operate within three overlapping systems; basal, modulated and stress induced glucocorticoids (recreated from Busch & Hayward 2009).

Basal

Basal glucocorticoid levels mediate basic requirements to sustain life e.g. basic glucose, salt and water regulation (Busch & Hayward 2009)

Modulated

Within the modulated range, glucocorticoid levels rise and fall in response to or in preparation for predictable changes in energetic demands at different times of day, seasonally and during different life history stages (Busch & Hayward 2009; Romero 2002).

For example, glucocorticoids are associated with diurnal and seasonal fluctuations in foraging activity (McEwen & Wingfield 2003; Romero 2002). Glucocorticoids peak in the period just before arousal from sleep, providing the high

levels of energy needed for increased locomotor activity, exploratory and food seeking behaviour (Sapolsky et al. 2000; Busch & Hayward 2009; Sheriff et al. 2011). Food intake is normally highest at the time of day when baseline glucocorticoids peak (Landys et al. 2006). Elevated glucocorticoids are also associated with prehibernatory foraging and fattening in many species including bats (Reeder et al. 2004a; Romero 2002).

Seasonal variations in glucocorticoids correlate with life-history stages that exert additional energetic demands on the organism (Landys et al. 2006; Romero 2002). Glucocorticoids increase during the breeding season, gestation and lactation (females) and mediate parental behaviour such as provisioning (Vitousek et al. 2014).

Energetically costly life history stages, such as breeding and raising offspring, occur when food resources are at their highest (Landys et al. 2006). Seasonal peaks in glucocorticoids, within the modulated range, serve the dual purpose of promoting foraging (and provisioning) behaviour at the same time as making these resources readily available within tissues (Busch & Hayward 2009; Romero 2002; Sapolsky et al. 2000). This is especially true of bat species that inhabit temperate regions with strong seasonality. Bats are adapted to give birth in spring, mate in autumn, and at the same time prepare for hibernation (Schofield & Mitchell-Jones 2011; Ransome 1990).

Stress related levels of glucocorticoids

The stress response is associated with heightened levels of glucocorticoids (Busch & Hayward 2009; Romero 2004). An acute increase in glucocorticoids, in response to a stressor, triggers a suite of adaptive physiological and behavioural changes known as the emergency life history stage (ELHS) that enables the organism to overcome the stressor (Table 4.1; Busch & Hayward 2009).

The key feature of the acute stress response is that all non-essential processes and behaviours are supressed until the threat has passed (Busch & Hayward 2009; Reeder & Kramer 2005). This includes foraging, behaviours related to breeding and digestion (Reeder & Kramer 2005; Sapolsky et al. 2000. Glucocorticoids promote behaviours that could be adaptive in the face of a threat such as irruptive behaviour, escape and avoidance, settlement in a new habitat and return to site post-disturbance (Busch et al. 2011; Reeder & Kramer 2005).

Glucocorticoids support the SNS to mount a flight-or-fight response by mobilizing energy stores from protein, synthesising glucose (gluconeogenesis) and promoting the availability of lipid energy from adipose stores (lipolysis; Sheriff et al. 2011; Reeder & Kramer 2005; Romero 2004). They also contribute to the cardiovascular stress response; e.g. elevated arterial pressure, heart rate and cardiac output (Reeder & Kramer 2005; Sapolsky et al. 2000). Glucocorticoids have a complex interaction with various components of the immune system, enhancing some aspects and inhibiting others (Sapolsky et al. 2000). Overall, it appears that whilst basal glucocorticoids mediate immune activation in the first moments of exposure to a stressor, elevated glucocorticoids eventually act to restrain the immune system (Sapolsky et al. 2000). Short-term elevated glucocorticoids are associated with enhanced memory formation, enabling the organism to learn from the experience; e.g. identify the threat more rapidly in future encounters, avoid re-encounters, and/or recall and repeat successful strategies for overcoming the threat (Sapolsky et al. 2000).

If the stressor is persistent or repetitive, glucocorticoid levels can remain high (but see section 4.3.11) and the organism is said to be under chronic stress (Romero 2004). Long-term exposure to heightened levels of glucocorticoids can have deleterious impacts on the organism's health (Table 4.1), survival and fitness that

1789 could potentially have negative consequences for the population (Wikelski & Cooke
1790 2006).

	Acute short-term stress response (minutes to hours)	**Chronic stress (hours to days)**
Energy mobilization	Cardiovascular activation; promotes protein metabolism, gluconeogenesis and lipolysis.	Insulin resistance, hyperglycemia, hypertension
Energy acquisition (foraging, feeding and digestion)	Suppression of feeding and foraging behaviour as well as digestion	Promotes deposition of fat and atherosclerotic plaques
Reproduction and associated behaviours	Temporary inhibition of reproductive behaviours; reduced territorial aggression	Sustained inhibition of reproductive behaviours; delay in onset of puberty
Neurological effects	Enhanced memory and learning	Neuronal cell death, cognitive impairment
Immune system	Restrains the immune system	Suppression of immune system, potentially to the point of collapse. Increased susceptibility to infectious disease.
Growth, tissue, healing	Energy is diverted away from peripheral tissues non-essential processes	Suppression of growth and metamorphosis, muscle and bone atrophy, poor wound healing, reduce body mass

Table 4.1 The physiological and behavioural changes mediated by glucocorticoids during an acute short-term stress response (adaptive) and as a result of prolonged, heightened levels in response to a chronic stressor (maladaptive). Collated from Landys et al. 2006; Reeder & Kramer 2005; Sapolsky et al.2000; Sheriff et al. 2011.

4.3.3 Regulation of the HPA axis: modification through experience

The influence of environmental stressors on glucocorticoids may depend on the duration of the stress as well as its intensity. Due to the negative impacts of long-term exposure to glucocorticoids, there are several mechanisms by which glucocorticoids levels can be attenuated; i.e. glucocorticoid levels are reduced, which may or may not be an indication that the organism no longer perceives the stimulus to be a threat. Here I will focus on modification of the HPA axis in adult organisms that contribute to reduced levels of glucocorticoids. However, the HPA axis can also be modified through maternal effects and during neonatal development (Romero 2004).

Hormonal habituation

Repeated or prolonged exposure to a novel stimulus can lead to hormonal habituation (Cyr & Romero 2009; Romero 2004). The organism may no longer perceive the stimulus as potentially harmful, possibly through learning. However, dishabituation may occur, where the organism shows a normal or exaggerated response to a novel stressor (Cyr & Romero 2009). Hormonal habituation does not incur or is not associated with physiological deterioration as with desensitization and exhaustion (Cyr & Romero 2009). Baseline levels are also unaffected.

Desensitization

On the contrary, desensitization alters the entire HPA axis as a result of continued or repeated challenge (Cyr & Romero 2009). The mechanisms for desensitization include downregulation of receptors, dysregulation of negative feedback, downregulation of hormone production, changes in concentration of synthetic enzymes, and changes in rates of hormone clearance (Cyr & Romero 2009; Romero 2004). Stress induced (and baseline) glucocorticoid levels may be reduced,

though the organism may not have concluded that the stimulus is innocuous (Cyr & Romero 2009). Thus, the animal may still experience stress despite reduced release of glucocorticoids.

Exhaustion

Exhaustion occurs when the organism can no longer support the energetic demands of the HPA axis and is essentially a complete breakdown of the HPA axis. In such cases, death may occur (Cyr & Romero 2009).

4.4 The conceptual framework, glucocorticoids and conservation research

Clearly, the significance of corticosteroid levels is potentially complicated for interpretation as a bioassay. Below, I present a conceptual framework that may aid this interpretation.

4.4.1 The concept of allostasis

McEwen and Wingfield (2003) provide a framework that integrates the different roles of glucocorticoids and how these three complimentary and overlapping processes influence each other. The model is widely recognised and has been cited over 1200 times (Web of Science 2018). Most importantly, this model provides an unambiguous definition of stress.

All organisms must maintain basic physiological systems that maintain life such as pH, body temperature, glucose levels and oxygen tension, i.e. homeostasis.

However, as the environment and/or life history change, set points (i.e. the optimal status of physiological systems) can also change. The process of maintaining homeostasis through change is referred to as “allostasis”. Glucocorticoids are one means by which allostasis is achieved (catecholamines and cytokines also have a role, but I will focus on glucocorticoids).

Glucocorticoids maintain allostasis by supporting physiological adjustments to shifting energetic require; ements made on the individual. Glucocorticoid levels contribute to the maintenance of the organism’s allostatic state and includes basal and modulated changes in glucocorticoid levels in response to predictable changes to the environment, seasonal energetic demands or life history stages as well as increases in response to unpredictable challenges.

The cumulative result of an organism’s allostatic state is the allostatic load. An organism’s allostatic load usually falls within a range that might be considered as coping, or not stressed, wherein allostasis is maintained. In the event of a disturbance that places an additional energetic demand on the organism, such as severe weather or human disturbance, the allostatic load can increase to such an extent that allostatic overload occurs (i.e. stress). McEwen and Wingfield describe two distinct types of allostatic overload defined by the availability of energy.

To illustrate this further see Figure 4.3. There are four key terms:

E_E: the minimum amount of energy required to maintain homeostasis (*Equilibrium*) at any life stage. This can change seasonally. For example, as temperature increases in spring and summer, the energy required to thermoregulate decreases for species adapted to temperate climes. Disease could also incur additional energetic demands to increase E_E.

E_I: the energy *invested* in maintaining homeostasis in response to predictable changes, i.e. regulated allostasis. E_I can change seasonally. For example, the energy required to maintain allostasis could increase during the breeding season or decrease during hibernation. The energy required to obtain food may decrease in spring and increase in winter. Disease or injury could also increase E_I as they increase the energy required to maintain allostasis and inhibit the ability of the organism to obtain sufficient resources to support allostasis.

E_G: the energy *gained* in the environment that is available to the animal to eat. At higher latitudes, this will vary seasonally, with potentially more food available in spring and summer when primary productivity is at its highest.

E_O: an additional event or disturbance that places an additional energetic demand (*ordeal*) on the organism such as increasing the energy required to obtain food. I have expanded this definition slightly from McEwen and Wingfield (2003) so as to include acute stressors that may place a short-term increased energetic demand on the organism (during which time foraging and feeding will have been suppressed as part of the ELHS) but do not necessarily affect the energy required to obtain food once the stress-inducing event has passed. For example, a psychological stimulus that elicits avoidance behaviour such as in the case of artificial lighting or moving vehicles.

Allostatic overload Type 1 occurs when energetic requirements exceed the current energy available to the organism. For example, a perturbation (E_O), such as a severe weather event, increases the energy required to obtain food over and above the energy available in the environment (i.e. $E_E + E_I + E_O > E_G$; Figure 4.3 a) or if $E_E + E_I$ increase as a result of disease or injury ($E_E + E_I < E_G$). The seasonal fluctuations of $E_E + E_I$ and E_G in relation to each other mean that a perturbation (E_O) may be more

likely to cause allostatic overload at certain times of the year than others. For example, a perturbation is more likely to cause allostatic overload in winter when $E_E + E_I$ are high and E_G low (Figure 4.3 b), than in summer when $E_E + E_I$ are low and E_G is high (Figure 4.3 c).

As E_O increases (i.e. as the severity and duration of a perturbation increases), glucocorticoids increase. If E_O exceeds E_G then an emergency life history stage (ELHS) is triggered (Figure 4.4). As described above, this includes suppression of all non-essential behaviours and processes such as reproduction and digestion. The physiological and behavioural changes during ELHS reduce allostatic load back below E_G and the organism has overcome the perturbation and the potential negative impacts of heightened levels of glucocorticoids in the body.

Allostatic overload type 2 occurs when a permanent or long-lasting challenge (E_O) increases allostatic load but does not exceed energy available in the environment (E_G). Glucocorticoids increase but not to an extent that triggers the ELHS. If the perturbation (E_O) passes, then glucocorticoids decline (Figure 4.5 a). If the perturbation persists, then glucocorticoids remain high and leads to allostatic overload type 2 (Figure 4.5 b). As described above, heightened levels of glucocorticoids have deleterious impacts on the organism.

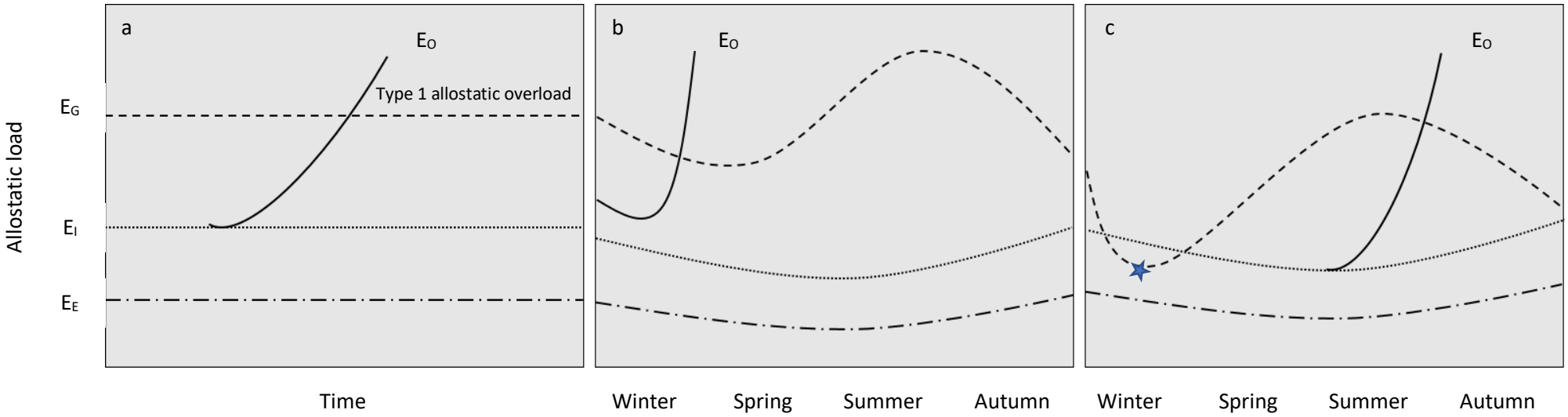


Figure 4.3 A framework for modelling energetic requirements of organisms during the course of a year, where E_E represents the minimum amount of energy required to maintain homeostasis (*Equilibrium*); E_I is the energy *invested* in maintaining homeostasis in response to predictable changes; E_G is the energy *gained* in the environment that is available to the animal to eat; and E_O represents an additional event or disturbance that places an additional energetic demand (*ordeal*) on the organism such as increasing the energy required to obtain food. Figure 4.3 a provides a simplistic hypothetical scenario where E_G, E_I and E_E remain constant over time. E_O represents an event (i.e. stimuli) that causes Type 1 allostatic overload (i.e. the stress response and the emergency life history stage). Figures 4.3. b and c include more realistic changes to E_G, E_I and E_E. Note that a stressful event (E_O) is more likely to trigger allostatic overload in winter. In winter, however, allostatic overload can be achieved if E_G drops below E_I (blue star, c). Adapted from McEwen and Wingfield (2003).

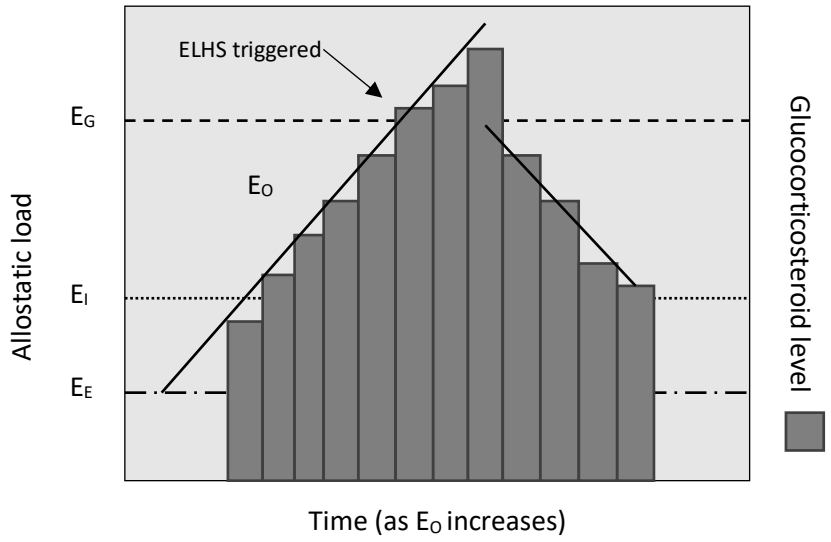


Figure 4.4 Summary of Type 1 allostatic overload. The emergency life history stage (ELHS) is trigger when $E_O + E_I + E_E$ exceed E_G. This causes the organism to modify its behaviour in order to rectify or avoid the cause of E_O which causes glucocorticoids to return to baseline levels. Adapted from McEwen and Wingfield (2003).

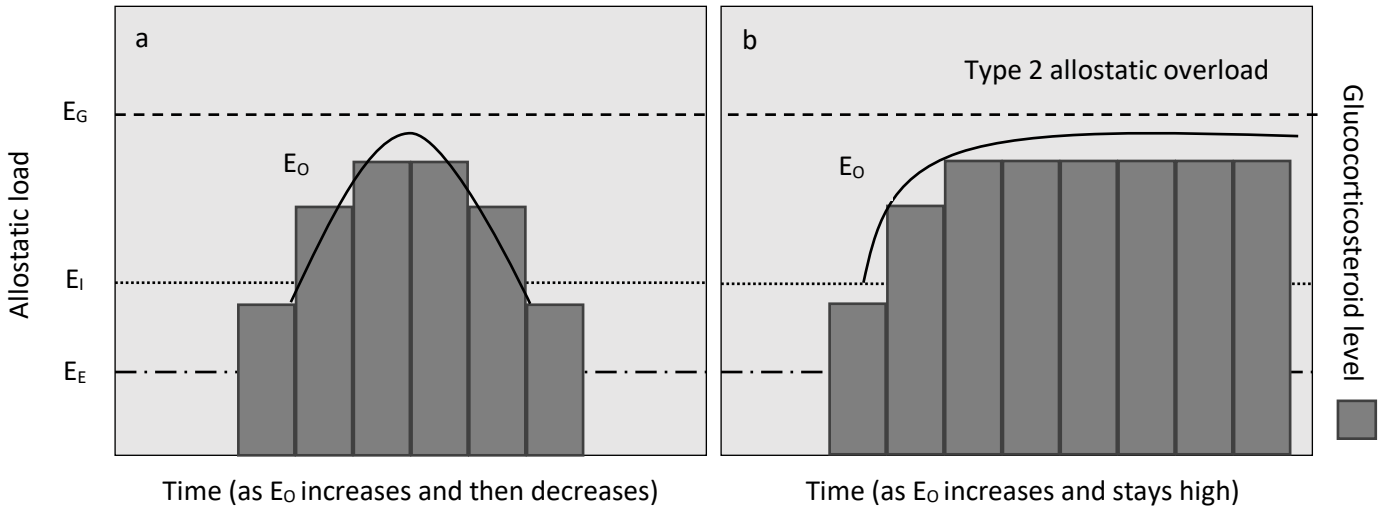


Figure 4.5 Two contrasting hypothetical scenarios where challenging conditions that incur an energetic cost (E_O) cause an increase in glucocorticoids. In the first scenario (a), the challenging conditions do not cause $E_O + E_I + E_E$ to exceed E_G and so allostatic overload Type 1 is not achieved. The challenging conditions subside, and glucocorticoids return to baseline levels. In the second scenario (b), the challenging

conditions also do not cause Type 1 allostatic overload. However, the challenging conditions do not subside, and so glucocorticoids remain high. This leads to allostatic overload Type 2. Adapted from McEwen and Wingfield (2003).

4.4.2 Allostatic load and overload in the context of conservation research

Elevated glucocorticoid levels are associated with a range of problems which may cause population declines. Several studies have shown that glucocorticoids are negatively correlated with body condition (Cayuela et al. 2017; Crino et al. 2013; Narayan et al. 2013), survival (Müllner et al. 2004) and reproductive success (Cyr & Romero 2007; Gobush et al. 2007; Strasser & Heath 2013; Vitousek et al.2014). For this reason, there is considerable interest in examining whether glucocorticoids correlate with measures of anthropogenic disturbance that are potential targets for conservation management (Figure 4.6; references are provided in Appendix 4.1) such as tourism, exposure to vehicles and anthropogenic habitat degradation, i.e. anthropogenic disturbances may be so severe that they have an impact at the population level and in so doing cause population declines, but that are also amenable to mitigation. Population level effects could express themselves much later than the initial commencement of the stressor, so a bioassay for stress would be a valuable early warning to initiate conservation intervention. The time it takes for population level effects to be expressed will depend on the type of stressor and aspects of the organism's ecology such as life span, age of sexual maturity, and typical reproductive rate (i.e. whether the organism is a K or R selected species). If research is able to link upstream measures (glucocorticoids) with downstream effects (e.g. population declines) via the stress response (Figure 4.7), it may be possible to: 1. Identify

1921 stressors; 2. use endocrinological bioassays to identify populations that are
1922 experiencing a stressor; and 3. predict and prevent a population decline.

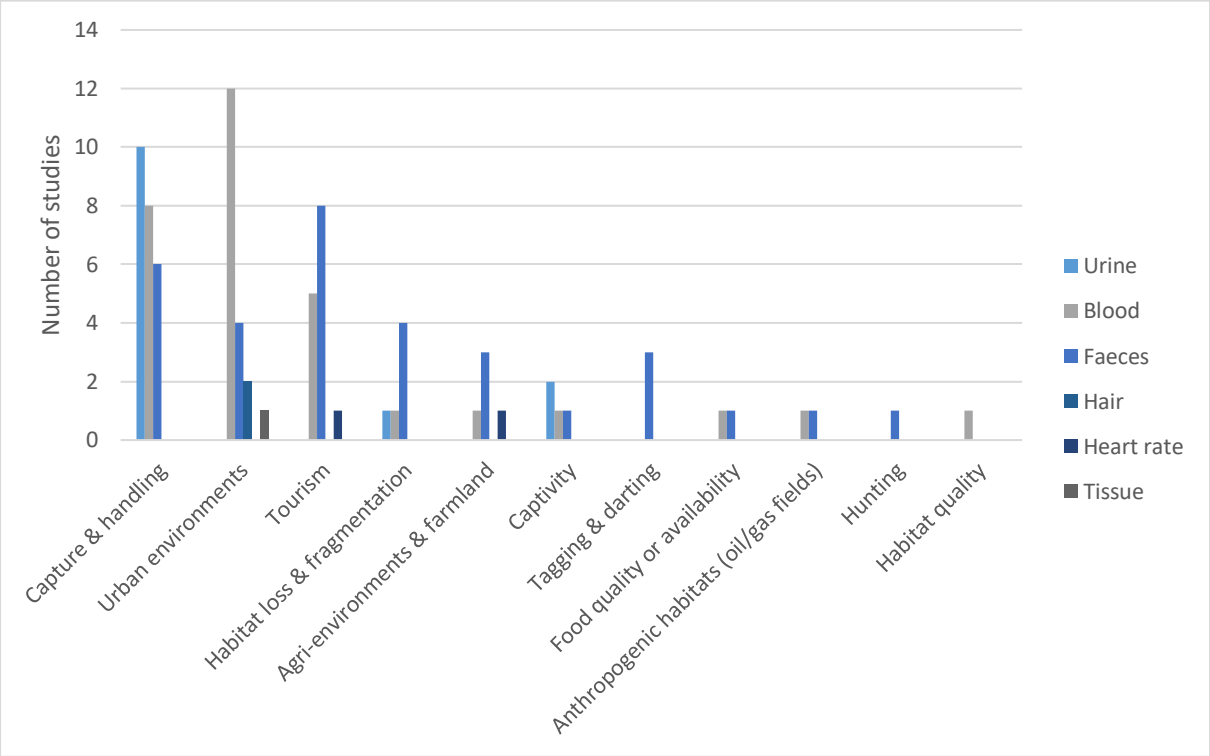


Figure 4.6 The number of studies investigating endocrinological responses to conservation relevant variables; 81 comparisons within 71 studies (see Appendix 4.1 for references).

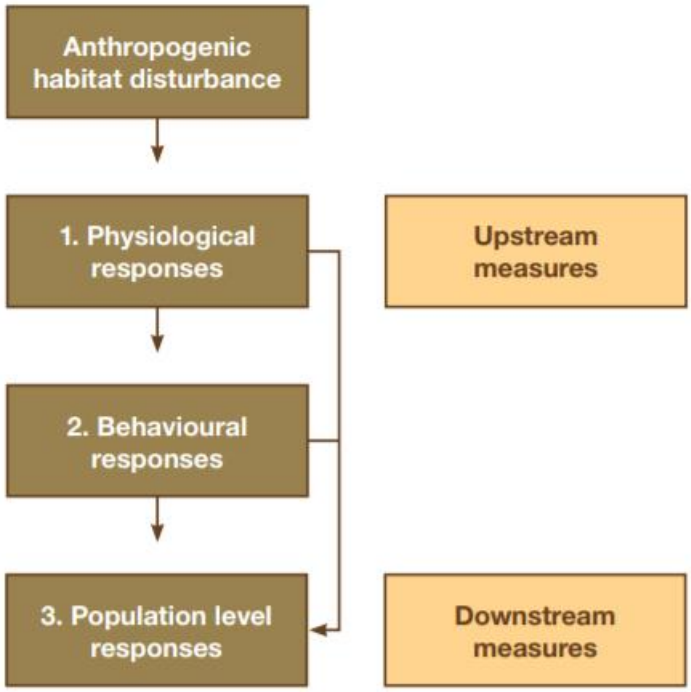


Figure 4.7 Exploring the impact of conservation relevant variables on HPA activity in wild animals could elucidate one mechanism by which population declines occur. (Reproduced from Fensome AG 2013).

Whilst much of the literature pertaining to anthropogenic disturbances frames its relevance in the context of acute short-term stress responses and chronic stress due to their direct influence on survival and fitness, it is also important to identify anthropogenic disturbances that may alter glucocorticoid levels without inducing stress or chronic stress, because these begin to reveal the basis of resilience. If common features of resilience can be identified among different species, a 'taxonomy of vulnerability' may eventually become possible.

The HPA axis incorporates three overlapping homeostatic systems, and as McEwen and Wingfield's model demonstrates, glucocorticoid levels within these three systems cumulatively contribute to the overall allostatic load. Anthropogenic

disturbances could contribute to allostatic load without causing allostatic overload yet render an organism vulnerable to additional perturbances that might normally have been within the capacity of the organism to cope. The endocrinal signature of allostatic load is a useful target.

For example, habitat loss or degradation as a result of human activities (e.g. logging, building) could reduce the availability of energy in the environment. The organism may attempt to compensate for this loss by investing energy into extending the period of time spent foraging or expanding the foraging area. If these behavioural changes do not enable the organism to compensate for the loss of energy in its immediate environment as well as the additional energy invested in accessing resources, allostatic load will increase. In the event of an additional perturbation, such as bad weather (e.g. drought further reduces the availability of food, or storms prevent the organism from foraging), allostatic overload may occur more readily.

Alternatively, if through behavioural changes the organism is able to compensate for the reduction of energy in the environment and the additional investment in foraging activity, there may be no apparent change in glucocorticoids. However, the organism is in a precarious situation. The environmental change has wrought behavioural changes that may balance the energy budget but expose the organism to additional potential risks. For example, more time spent foraging means more time exposed to predators and perhaps less time spent nest guarding. Expanding the range within which the organism forages could increase the frequency with which territorial encounters occur with conspecifics, or the frequency of crossing roads in turn increasing the risk of collision with vehicles. I highlight this issue as an example of the importance of interpreting glucocorticoid information in the context of behavioural information, without which one might conclude that there is no causal link

between an anthropogenic disturbance and downstream outcomes. In this example, the endocrinal signature of increased foraging may be a useful hint that the individual bat is more vulnerable to collision mortality.

The McEwen and Wingfield model also highlights the importance of studying the cumulative impact of multiple anthropogenic disturbances on allostatic load. Whilst it is important (though very difficult) to identify and compare singular stressors (e.g. artificial lighting or habitat loss) in order to make conservation decisions, animals in the wild are exposed to multiple sources of stress of varying severity, duration and they may or may not overlap in time. Hence, the cumulative influence of multiple stressors and the combination of those stressors may have more pronounced impacts on an organism's allostatic load than an understanding of the organism's stress response to each individual stressor might imply. For example, by studying the impacts of artificial lighting on glucocorticoids we might conclude that an organism can habituate over time. Separately we consider the impact of vehicles and conclude that they cause a moderate stress response. But together we might find that organisms habituated to artificial lighting have an exaggerated stress response to moving traffic; i.e. dishabituation has occurred. This constitutes both an advantage and a challenge to the use of endocrinological bioassays in conservation. On the one hand, they may present an integrated stress status of the organism, but on the other hand it may be difficult to identify particular stressors that are targets for mitigation efforts.

4.4.3 Using faecal glucocorticoid measurements in conservation research: potential benefits and drawbacks

Benefits

Faecal endocrinology in general (and glucocorticoid analysis in particular) offers a potentially non-invasive means of assessing baseline glucocorticoid levels in an individual. Analysis of glucocorticoids in fur, feathers and tissue all require capture and handling of individual animals which may not be practical, ethical or legal for certain species (Sheriff et al. 2011). In addition, quantifying glucocorticoids in blood samples not only requires the capture and handling of individuals but samples must be taken within 3 minutes to avoid the confounding effect of quantifying handling stress (Millspaugh & Wasburn 2004).

Corticosteroids and their metabolites extracted from faecal samples reflect basal levels, modulated cycles and allostatic overload (stress related) glucocorticoid levels; i.e. it provides an integrated quantification of an organism's circulating glucocorticoids over a longer period of time (hours to days; Reeder & Kramer 2005; Sheriff et al. 2011).

Moreover, faecal measures capture "free" hormones. In many species, a proportion of the circulating glucocorticoids are bound to corticosteroid binding globulin (CBG; Reeder and Kramer 2005). There are two hypotheses regarding the purpose of this system; the 'carrier' hypothesis and the 'buffer' hypothesis (Reeder and Kramer 2005). Under the "buffer hypothesis", CBG serves to reduce the negative impacts of glucocorticoids on tissues whereas under the "carrier hypothesis" CBG acts as a transport molecule, delivering glucocorticoids to tissues and potentially mediating their actions (Reeder and Kramer 2005). According to the "free hormone hypothesis", it is

the proportion of unbound glucocorticoids that are active and indicate the organisms allostatic state (Sheriff et al. 2011)

As such quantifying faecal glucocorticoid levels is a useful tool for assessing the impact of different types of stressor, including long-term and short-term changes to the environment or exposure to other noxious stimuli.

Drawbacks

The complex biology of the interaction between an organism and its environment mean that various confounding factors can obscure the effects of certain stressors of interest. In effect, the system may be rather noisy as a bioassay. For example, as noted above, glucocorticoids can be reabsorbed in the gut. Consequently, differences in diet (such as the amount of fibre) between individuals, during an individual's lifetime, or between populations, could introduce uncontrolled variation (Goyman 2012).

The proportion and structure of metabolized glucocorticoids that are excreted in urine and faeces differs among species and can also differ between males and females (Sheriff et al. 2011). Differences in the microbial ecosystem in the gut and their action on glucocorticoids and their metabolites may also contribute to differences between individuals and species (Goyman 2012)

Post-excretion, there are two potential factors that could affect yields, relating to microbial and enzymatic activity. Simulated rainfall has been shown to increase yield, possibly as it relates to bacterial and microbial activity (Millspaugh & Washburn 2004; Washburn & Millspaugh 2002).

The influence of temperature on FGM yield is more nuanced; steady temperatures (room temperature 22°C and high temperatures 38°C) do not appear to

increase yield. However, alternating room temperature and freezing temperatures does increase yield. This is primarily relevant to post collection handling and storage of samples, but in some field locations, large fluctuations in temperature during the night and day could affect FCM yield if more than 12-24 hours have passed between excretion and collection (Washburn and Millspaugh 2002).

In summary, it may be necessary to understand the influence of quite a large range of covariate factors before it is possible to interpret differences in corticosteroid levels between individuals or populations.

4.5 The impact of roads on steroids in bats

4.5.1 Introduction

In order to begin to use faecal endocrinology as an indicator of anthropogenic disturbance, it is necessary to make a credible case that the particular kind of disturbance could plausibly increase corticosteroids, either as part of a stress response (allostatic overload) or by reducing the availability of resources (increasing allostatic load). Below, I begin to make this case for the affect of roads on bats. I therefore conducted a systematic review of the literature (Table 4.2; see Appendix 4.2 for search terms, method and references) pertaining to the impact of roads on glucocorticoid levels in wild living animals. My review (Table 4.2) reveals that roads have an impact on baseline glucocorticoid levels in amphibians (references 12, 15), birds (references 2, 5, 6, 10, 13, 16, 18), mammals (references 3, 4, 7, 14) and reptiles (9). Below, I discuss our current understanding of the specific mechanisms by which roads can act as stressors on animals in general.

Road related variable	Effect on baseline glucocorticoid levels			Research effort
	Increase/higher	No effect	Decrease/lower	
Traffic noise	[2] [6] [12] [18]	[16] [17] [18]		7
Proximity to road	[5] [13] [14]	[6] [7] [11]		6
Number of vehicles	[4] [4]	[7]	[9] [15]	5
Road impacted site versus control		[1] [8] [9]		3
Comparison of exposure to different road types (e.g. major versus minor)	[3] [7]			2
Presence of vehicles	[4]			1
Length of roads within territory		[11]		1
Principle component incorporating number of vehicles, speed of vehicles, number of lanes and area of human development.	[10]			1
Total number of effects	13	11	2	

Table 4.2 The effect of road related variables (total = 8) on baseline glucocorticoid levels reported in 18 studies (references in Appendix 4.2). Out of a total of 26 correlations between baseline glucocorticoid levels in wild living animals and road related variables or comparisons between individuals exposed to road related variables and controls, 13 were associated with an increase or higher glucocorticoids, 11 reported no effect and just two reported a negative correlation. Research effort refers to the total number of times each variable has been reported. A single study (4) reported the same effect for two different species; wolves (*Canis lupus*) and elk (*Cervus elaphus*).

Traffic noise is the most commonly studied road related variable (six studies) and was found to increase glucocorticoids in four out of seven comparisons. Proximity to the road is positively correlated with glucocorticoid levels in three out of six studies (Table 4.2). Proximity to the road has mostly been investigated in relation to nesting birds (references 5, 6, 11, 13 in Table 4.2) but also grazing impala (*Aepyceros melampus*, 7 in Table 4.2) and wood mice (*Apodemus sylvaticus*, 14 in Table 4.2). The number of vehicles or traffic density correlates with glucocorticoid levels in four out of five reported correlations (two positive correlations and two negative correlations) whilst major roads are consistently associated with higher glucocorticoid levels than minor roads.

Of the nine studies that reported at least one non-significant effect of a road related variable on glucocorticoid levels, four studies (references 6, 7, 9, 18 in Table 4.2) reported at least one significant correlation with a different road related variable. Of the five studies that found no effect of road related variables on baseline glucocorticoid levels at all (references 1, 8, 11, 16, 17 in Table 4.2), two observed that the focal species is often found in anthropogenic environments and may therefore be less sensitive to road-related stressors (references 1, 17 in Table 4.2) and another suggested that the focal species may benefit from additional foraging opportunities in roadside edge habitats (reference 8 in Table 4.2).

Not included in Table 4.2 are results from three studies that examined experimentally induced stress responses (Angelier et al. 2015; Crino et al. 2013; Owen et al. 2014). These studies compare the difference between an organism's baseline glucocorticoid levels and its glucocorticoid levels during an experimentally induced stress response. Stress is usually induced through a handling and restraint protocol. The ability of individuals to mount an acute stress response is either compared

between individuals exposed (or not) to road related variables (e.g. a comparison of individuals exposed to traffic noise compared to controls) or correlated with road related variables (e.g. the density of traffic).

Of the three studies investigating experimentally induced stress responses, two compared the stress response between nestlings that had and had not (controls) been exposed to traffic noise (Angelier et al. 2015; Crino et al. 2013), and one study compared the stress response in snakes (*Agkistrodon contortix*) caught on roads versus individuals that were caught in forests (Owen et al. 2014). This study also correlated individual stress responses with the number of vehicles recorded on the road (i.e. traffic density) on the night the individual was caught. Traffic noise (Crino et al. 2013), road crossing and traffic density (Owen et al. 2014) were all associated with a dampened stress response, i.e. individuals exposed to roads and traffic are less able to mount an acute stress response. The dampened stress response may be a sign of modification of the HPA axis in response to roads (e.g. habituation, desensitization or exhaustion). Conversely, snakes found on roads may be self-selecting, i.e. they are on roads because they have a downregulated stress response in the first place and consequently are less sensitive to stressors such as vehicular traffic which might induce avoidance behaviour in snakes with stronger stress responses. The single study that found no effect of a road related variable (traffic noise) on the stress response (Angelier et al. 2015), had investigated the effect of traffic noise on a successful urban exploiter, the house sparrow (*Passer domesticus*).

To date, most studies have focused on the impact of roads on glucocorticoid levels in birds (Figure 4.8). Just four of the studies included in Table 4.2 have explored the impact of roads on mammals, including impala (*Aepyceros melampus*, 7), squirrel-gliders (*Petaurus norfolcensis*, 3), wood mice (*Apodemus sylvaticus*, 15), and wolves

(*Canis lupus*) and elk (*Cervus elaphus*, 4) and no studies have been conducted on the impact of roads on bats.

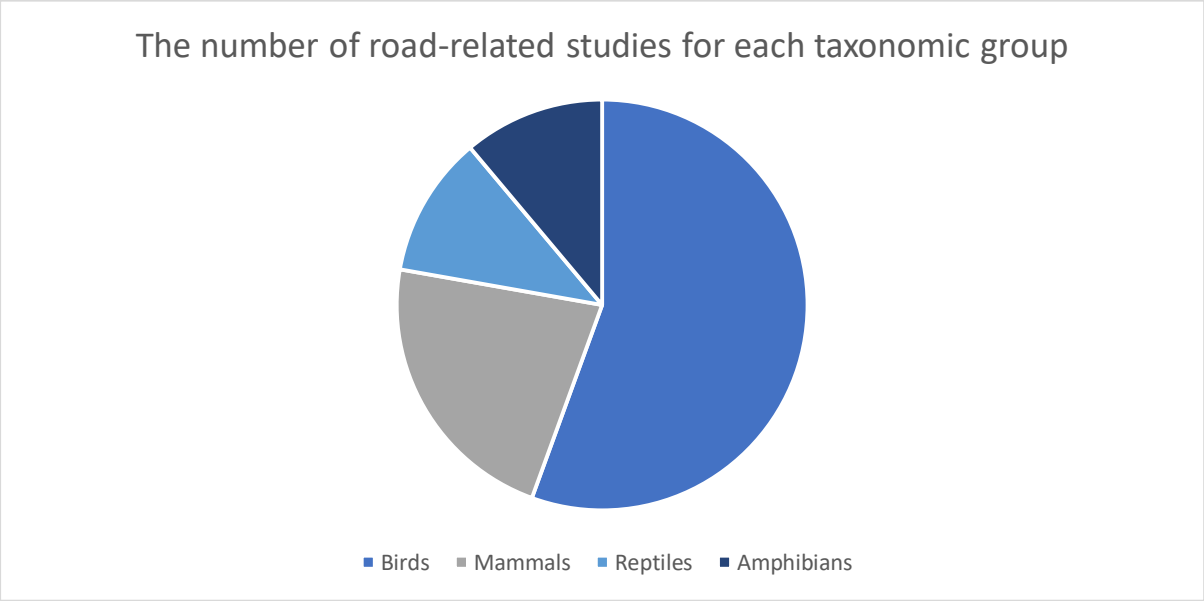


Figure 4.8 The number of studies that have explored the impact of road related variables on glucocorticoids in wild living species by taxonomic group; birds = 10, mammals = 4, reptiles = 2, amphibians = 2, total = 18).

In summary, a review of the research to date relating to the impact of roads on glucocorticoids in wild animals indicates that:

a) Roads have an impact on glucocorticoids across taxonomic groups, but no studies have investigated the impact of roads on bats.
b) A range of road related variables ought to be included in any study investigating roads as several studies demonstrate that glucocorticoid levels may be associated with certain road-related variables and not others.

c) Most of the road related variables investigated to date focus on the potential for roads to act as psychological stressors (e.g. vehicular traffic; i.e. E_O) rather than their potential to modify the environment to an extent that the organism struggles to maintain allostasis due to a lack of resources (e.g. road density or habitat quality, but see references 3, 7, 10, 11 in Appendix 4.2; i.e. E_G, E_I). Although, the potential for road related variables to act as psychological stressors, or to reduce the availability of energy in the environment, or to impose additional energetic demands on the individual, are not mutually exclusive.

d) In the few studies where no effect at all has been reported, the authors point to the fact that the study species is known to either live in or exploit anthropogenic or urban areas (references 1, 8 in Appendix 4.2).

However, while our general understanding is fairly well advanced, our knowledge about the effects of roads on levels of stress in bats is much less well developed. Given that some species of bat are known to avoid roads and/or characteristics associated with roads such as artificial lighting and loud background noise (Chapter 2; Fensome & Mathews 2016), bats are therefore an ideal, as well as novel, study organism for investigating a comprehensive range of road-related and habitat quality variables on glucocorticoid levels. Further, the habitat requirements of bats are such that we might expect that they are sensitive to habitat degradation or reduction by roads. Bats forage within relatively large areas given their small body size in order to exploit ephemeral invertebrate prey. There are many ways in which roads could potentially reduce the availability or alter the distribution of insect prey, or alter the behaviour of foraging bats, and thereby have an affect on the availability of energy in the environment (E_G) or the energy invested by bats (E_I) in order to maintain allostasis.

As the first test of the feasibility and utility of faecal endocrinology to investigate the impact of roads on bat roosts, I will report a correlative study investigating the impact of roads on corticosteroid levels exhibited collectively within maternity roosts. I collected faecal samples from six maternity roosts of two species, *Rhinolophus hipposideros* and *Plecotus auritus*, in order to examine whether a range of endocrinological compounds correlated with:

1. The distance of the roost to the nearest road (A, B, minor and all road types combined)
2. The density of different types of road (A, B, minor and all road types combined) within the core sustenance zone.
3. The area of the patch defined by major roads (A and B roads) in which the roost is situated.
4. The percentage of broadleaved woodland and built environments within the core sustenance zone.
5. The percentage of broadleaved woodland and built environments within the road-defined patch.
6. Weather conditions including temperature (average and average minimum), wind speed and average rain events.

Before presenting the details of the study, I first provide an introduction to the study species and their general ecology.

4.5.2 Study species: *Rhinolophus hipposideros* and *Plecotus auritus*

Both *R. hipposideros* and *P. auritus* are low flying, clutter adapted species that typically forage within woodland and along woodland edges. Bat species with these

traits are more likely to avoid roads (Chapter 2, Fensome & Mathews 2016). Indeed, *P. auritus* has been shown to cross roads less frequently than open-adapted and high-flying species (Abbott et al. 2012). This suggests that both species may be sensitive to stimuli associated with roads (such as vehicles or artificial lighting) that elicit a stress response and avoidance behaviour. Moreover, if *R. hipposideros* and *P. auritus* avoid roads, they are more likely to be limited in their foraging opportunities, potentially restricted to foraging within areas bordered by roads.

4.5.3 Study group: Maternity roosts

As McEwen and Wingfield's model demonstrates, basal and modulated glucocorticoid levels contributing to allostatic load influence whether and to what extent glucocorticoids rise in response to additional stimuli or environmental disturbances, i.e. these background, seasonal and predictable changes in glucocorticoids can influence an organism's vulnerability to allostatic overload (stress).

Pregnant and lactating female bats may be more sensitive to changes in their environment that effect energetic availability (e.g. road density) because they already have a higher allostatic load due to the demands of pregnancy and lactation. As evidence of this, when baseline and stress induced glucocorticoid levels are compared between non-reproductive males and females, there is frequently no difference (Table 4.3 and references therein). However, pregnant and lactating females tend to have both higher baseline and stress induced glucocorticoid levels than males (Allen et al. 2010; Klose et al. 2006; Reeder et al. 2004a; Reeder et al. 2004b).

Taken together, the potential vulnerability of *R. hiposideros* and *P auritus* to the impacts of roads, and the sensitivity of pregnant and lactating females in maternity

roosts, suggests that this is an ideal study system with which to investigate the impact of roads on bats.

Species	Hormone	Was there a difference in glucocorticoid levels between males and females?		Reference
Baseline measures				
Desmodus rotundus	Cortisol	No difference between non-reproductive adults		Lewanzik et al. 2012
Carollia perspicillata	Cortisol	No difference between non-reproductive adults		Lewanzik et al. 2012
Tadarida brasiliensis	Cortisol	No difference between non-reproductive adults		Allen et al. 2010
Pteropus vampyrus	Cortisol	No difference between non-reproductive adults		Widmeir & Kunz 1993
Artibeus jamaicensis	Corticosterone	No difference between non-reproductive adults		Klose et al. 2006
Eptesicus isabellinus	Glucocorticoids	Sex had no effect on FGM concentrations, but dataset only included a few males		Kelm et al. 2015
Myotis lucifugus	Cortisol	Males had similar levels to females in early pregnancy and lactating females	P & L	Reeder et al. 2004a
Myotis lucifugus	Corticosterone	Males had similar levels to females in early pregnancy and lactating females	P & L	Reeder et al. 2004a
Myotis lucifugus	Cortisol	Females in mid-to-late pregnancy had higher levels than males.	P	Reeder et al. 2004a
Myotis lucifugus	Corticosterone	Females in mid-to-late pregnancy had higher levels than males.	P	Reeder et al. 2004a
Myotis lucifugus	Cortisol	Prior to hibernation females had higher levels than males	H	Reeder et al. 2004a
Tadarida brasiliensis	Cortisol	Gestating and lactating females tended to have higher levels compared to non-reproductive males and females	P & L	Allen et al. 2010

Pteropus hypomelanus	Corticosterone	Pregnant females had higher corticosterone levels than males	P	Reeder et al. 2004b
Pteropus hypomelanus	Cortisol	Males consistently had higher cortisol levels than females through the year	P & L	Reeder et al. 2004b
Pteropus hypomelanus	Corticosterone	Males had similar or higher corticosterone levels than females except when females were pregnant	L	Reeder et al. 2004b
Pteropus hypomelanus	Glucocorticoids	Males consistently had higher total glucocorticoids than females through the year	P & L	Reeder et al. 2004b
Pteropus hypomelanus	Glucocorticoids	Glucocorticoid levels were two times greater in non-reproductive males than non-reproductive females		Widmeir & Kunz 1993
Stress induced measures				
Desmodus rotundus	Cortisol	No difference between non-reproductive adults		Lewanzik et al. 2012
Carollia perspicillata	Cortisol	No difference between non-reproductive adults		Lewanzik et al. 2012
Artibeus jamaicensis	Corticosterone	No difference between non-reproductive adults		Klose et al. 2006
Myotis lucifugus	Corticosterone	No difference between males and pregnant and lactating females	P & L	Reeder et al. 2004a
Myotis lucifugus	Cortisol	Pregnant and lactating females had higher levels than males	P & L	Reeder et al. 2004a
Artibeus jamaicensis	Corticosterone	Reproductively active females have a stronger stress response than males in the first 60 minutes following the handling and restraint protocol.	P & L	Klose et al. 2006
Pteropus hypomelanus	Corticosterone	Females in late pregnancy had a stronger stress response than males	P	Reeder et al. 2004b
Pteropus hypomelanus	Cortisol	Males had a stronger stress response than females except when females were in late pregnancy.	L	Reeder et al. 2004b

Pteropus hypomelanus	Corticosterone	Males had a stronger stress response than females except when females were in late pregnancy.	L	Reeder et al. 2004b
Pteropus hypomelanus	Glucocorticoids	Males had a stronger stress response than females except when females were in late pregnancy.	L	Reeder et al. 2004b

Table 4.3 Twenty-six comparisons between male and female baseline and stress-induced glucocorticoid levels reported in eight species from eight studies. All individual comparisons are reported for cortisol and corticosterone. Where authors compared combined cortisol and corticosterone levels, I have referred to them as glucocorticoids. Pink squares denote that that females had higher glucocorticoid levels, blue denotes males had higher glucocorticoid levels and white squares denote no significant difference between sexes. The letter P denotes a comparison between males and pregnant females, the letter L denotes a comparison between males and lactating females and the letter H denotes a comparison between males and females preparing for hibernation.

4.5.4 Endocrinological targets

As well as glucocorticoids (including cortisol and corticosterone, the primary "stress hormones"), faecal samples were assessed for concentrations of closely related corticosteroids (e.g. cortisone, aldosterone and 11-deoxycorticosterone), that also regulate the stress response within the HPA axis (Balm 1999) as well as their precursors and metabolites (see Appendix 4.5). Cortisol and corticosterone have previously been measured in bats (Allen et al. 2010; Gustafson & Belt 1980; Kelm et al. 2015; Klose et al. 2006; Lewanzik et al. 2012; Reeder et al. 2004a; Reeder et al. 2004b) as has hydrocortisone and deoxycorticosterone (Kelm et al. 2015).

Whilst many studies investigating the impact of anthropogenic disturbance assess faecal corticosteroid metabolites, there are precedents for assessing unmetabolized corticosteroid hormones (Tempel and Gutierrez 2004; Wasser et al 1997; Creel et al. 2002).

Faecal samples were also assessed for concentrations of reproductive hormones (e.g. estrone, testosterone). The anti-reproductive effects of heightened glucocorticoid levels is well documented (Balm 1999, Sapolsky 2000). In mammals, glucocorticoids contribute to the decline in circulating sex steroid concentrations (Sapolsky et al. 2000) Further, cortisol suppresses the release of oestrogens (estrone, estradiol and estriol) and progesterone (Balm 1999)

Corticosteroids should be negatively correlated with reproductive hormones therefore. Wherever a positive correlation between stress related compounds and an environmental variable is demonstrated, it is predicted that there will be a negative correlation between the same environmental variable and sex related hormones. Conversely, wherever a negative correlation between corticosteroids and an

environmental variable is predicted, reproductive hormones are expected to positively correlate with that environmental variable.

4.6 Predictions: allostatic load and overload in the context of roads

McEwen and Wingfield's concept of allostasis provides a useful framework for envisioning and predicting how roads could have an impact on corticosteroid levels in bats through several pathways, and how these different pathways could have a cumulative impact on allostatic load (Table 4.4).

4.6.1 Proximity of roosts to roads

Faecal corticosteroid levels are predicted to negatively correlate with the proximity of roosts to roads. Proximity to the road is negatively correlated with baseline corticosteroid levels in three of six previous studies (Table 4.2). There are a number of possible causes of this association, as follows.

Individuals living in roosts closer to roads may be exposed to louder traffic noise and encounter vehicles more frequently (i.e. more E_O events; Table 4.4). The acoustic volume of traffic is associated with an increased likelihood of road avoidance behaviour in bats. Traffic noise is much louder than naturally occurring acoustic stimuli and could induce a stress response as a novel stimulus or in response to discomfort in animals with acute hearing. It is possible that traffic noise masks acoustic cues associated with predators (Hayward et al. 2011; Strasser & Heath 2013) which leads to hypervigilance on the part of bats mediated by a stress response. Traffic noise was

associated with elevated glucocorticoid levels in four out of seven comparisons (Table 4.2).

Moving vehicles could be perceived as direct threats and elicit predator avoidance behaviour (Lian et al. 2011). Road avoidance behaviour in bats is associated with vehicle presence (Fensome and Mathews 2016; Chapter 2).

Bats living closer to roads are more likely to be exposed to artificial lighting. Some species of bat, including *R. hipposideros*, avoid artificial lighting (Stone et al. 2009; Stone et al. 2015) and it is possible that there may be a stress response mediating such avoidance behaviour. In terrestrial vertebrates, artificial lighting is associated with higher glucocorticoids in birds (Russ et al. 2015; Ouyang et al. 2015), mice (Fonken et al. 2009).

4.6.2 Road density

Faecal corticosteroid levels are predicted to correlate positively with the density of roads within the core sustenance zone (Table 4.4) for the following reasons.

Road density is likely to be negatively correlated with the total area of woodland within the CSZ as a result of direct habitat loss. Both *R. hipposideros* and *P. auritus* are insectivorous and forage in woodland. Woodland habitat loss associated with roads could be responsible for a reduction in food availability (i.e. road density could be negatively correlated with E_G). Baseline FGMs have been shown to negatively correlate with food abundance (Busch & Hayward 2009).

The greater the density of roads within CSZs, the more likely it is that woodland is fragmented. Even if fragmentation is not associated with a reduction in food

abundance, the altered distribution of insect prey as a result of habitat loss and fragmentation could force bats to invest more time and energy in foraging (i.e. increases E_I). Alternatively, if insect abundance is highest in edge habitats as opposed to core woodland, fragmentation could increase foraging opportunities by increasing the proportion of edge habitat relative to core habitat (i.e. reduce E_I). Fragmentation is associated with higher glucocorticoid levels in bats (Seltmann et al. 2017) and lemurs (*Eulemur collaris*; Balestri et al. 2014) but not in black and gold howler monkey (*Alouatta caraya*; Cantarelli et al. 2016). Fragment size did not influence FGM levels in either red howler monkeys (*Allouatta seniculus*) or brown spider monkeys (*Ateles hybridus*; Rimbach et al. 2013).

The greater the density of roads within the CSZ, the more frequently bats are likely to encounter moving vehicles, traffic noise and artificial lighting (i.e. E_O events increase). Widespread artificial lighting along roads could enhance the barrier effect of roads for light sensitive bat species, thereby reducing the availability of foraging space (reduce E_G).

Traffic noise could have a similar effect. In *Myotis myotis* (the greater mouse-eared bat), traffic noise reduces foraging activity (Schaub et al. 2008) and efficiency (Siemers and Schaub 2010). Traffic noise could impair the ability of bats to forage (reduce E_G, increase E_I) by masking the sound of insect prey (Schaub et al. 2008; Siemers and Schaub 2010).

4.6.3 Road type

Major roads should have a stronger effect than minor roads on corticosteroid levels both in terms of proximity to a roost and density within the CSZ. Major roads

tend to have more traffic and are more likely to be artificially lit (i.e. more E_O events). Major roads also tend to be wider. Consequently, major roads have a stronger barrier effect from the perspective of bats (Chapter 2, Fensome & Mathews 2016) which may have an effect on glucocorticoid levels if they reduce access to foraging areas and/or increase the energy invested in foraging (reduce E_G, increase E_I). Road width was one of the road related variables within a principle component describing anthropogenic disturbance and correlated positively with glucocorticoids (Strasser & Heath 2013, see also Table 4.2). Major roads have been shown to have a greater impact on glucocorticoids than minor roads in the two studies where this has been studied (Brearley et al. 2012; Lunde et al. 2016; Table 4.2).

4.6.4 Road-defined fragmentation and the effects of patch size

Faecal corticosteroid levels could be negatively correlated with patch size (Ellis et al. 2012) as defined by major roads for two reasons. First, if major roads act as a partial or complete barrier to landscape scale movement, patch size defined by major roads determines the availability of resources (i.e. E_G is positively correlated with road defined patch size). Second, as patch size decreases the greatest distance a roost can be from a road also decreases. Road-defined patch size, therefore, could also exert an influence on stress levels by proximity to stressors such as traffic noise (i.e. E_O events increase, related to proximity of roosts to roads above).

4.6.5 Habitat quality

Faecal corticosteroid levels are predicted to negatively correlate with the availability of broadleaved woodland. A greater area of broadleaved woodland may

provide more foraging opportunities for species adapted to foraging in woodlands (i.e. greater E_G).

By correlating corticosteroids against both the proportion of broadleaved woodland in the CSZ and within the road-defined patch, it may be possible to determine which environmental factor has the strongest influence. A stronger correlation with road-defined patch size would suggest that bats preferentially forage within the road defined patch and avoid crossing roads.

Corticosteroid levels are predicted to positively correlate with the proportion of built environments within the core sustenance zone or road-defined patch. Built environments are associated with artificial lighting (E_O, E_G and E_I) and are likely to be negatively correlated with the availability of suitable foraging habitats such as broadleaved woodland (E_G).

4.6.6 Weather conditions as a possible confounding factor

The influence of roads could be obscured by other factors. So, for example, weather conditions influence the amount of time bats spend foraging (Cyr & Romero 2009). Bats are less likely to emerge from the roost during or shortly after rain, when temperatures are low or in windy conditions (Kunz & Fenton 2003; Voigt et al. 2011). Weather conditions could also influence the availability of insect prey; insects may be less active in cold and rainy conditions. Rain could also impose additional energetic costs; flight is more energetically costly for wet bats (Lewanzik et al. 2012 and references therein; Voigt et al. 2011). Temperature could influence energetic expenditure related to thermoregulation; higher ambient temperatures up to 30 are associated with lower relative use of respiratory energy (Ransome 1990).

Thus, faecal corticosteroids are predicted to correlate positively with average wind speed and rainfall events (by reducing E_G and increasing E_I) but negatively correlated with average temperature and average minimum temperature (increasing E_G, decreasing E_I and E_E).

Variable	Direction of correlation		Effect on Energy terms			
	FGMs	RH	E_G	E_I	E_E	E_O
Road related variables						
Road density (all)	+	-	↓	↑		↑
A	+	-	↓	↑		↑
B	+	-	↓	↑		↑
Minor	+	-	↓	↑		↑
Proximity of roost to road (all)	+	-				↑
A	+	-				↑
B	+	-				↑
Minor	+	-				↑
Road defined patch size	-	+	↑	↓		↓
Habitat quality						
Broadleaved woodland in the CSZ (%)	-	+	↑			
Broadleaved woodland in the road defined patch (%)	-	+	↑			
Built environment within the CSZ (%)	+	-	↓			↑
Built environment within the road defined patch (%)	+	-	↓			↑
Weather conditions						
Average wind speed	+	-	↓			
Average rainfall events	+	-	↓	↑		
Average temperature	-	+	↑	↓	↓	
Average minimum temperature	-	+	↑	↓	↓	
Total = 17	+ 12	- 5	+ 5	- 12	↑ 5	↓ 8	↑ 15	↓ 6

Table 4.4 The predicted direction of correlations between faecal corticosteroids and reproductive hormones (RH) for each of 17 predictor variables, including 9 directly related to roads, 4 indirectly related to roads (habitat quality) and 4 related to weather conditions. The basis for each prediction as a consequence of each variable's influence on the energy available in the environment (E_G), the energy invested in accessing resources (E_I), the minimum energy required to maintain homeostasis (E_E) or the potential for additional perturbances such as psychological stimuli from a perceived threat from vehicles or artificial lighting (E_O) are also noted.

4.7 Methods used in the correlative study

4.7.1 Collection of faecal samples

Six maternity roosts (*Rhinolophus hipposideros* n=4; *Plecotus auritus* n=2) were visited between June and September 2015. Roosts were visited up to four times each as part of a lighting experiment presented in section 4.9.

In order to examine whether roads or other environmental factors influence corticosteroids or reproductive hormones excreted in the faeces of female bats, the analysis presented here is based on pre-treatment or baseline samples, i.e. the first batch of faecal pellets collected from each roost after one visit. These were collected between June and August 2015.

The majority of individuals in maternity roosts were pregnant females or females rearing young. Any males in the roost were likely to have been neonates or juveniles. Glucocorticoids and reproductive hormones increase with age and males and females can have significantly different glucocorticoid levels. We can assume with

some confidence that the endocrinological compounds measured in this study (and the following experimental study) come from adult females and not from adult males. Neonates of either sex (and presumably there is a roughly equal sex ratio) are very likely to have 1. very small droppings and 2. very low levels of glucocorticoids.

Roosts were entered after dark at approximately 10pm when most bats had left the roost. Plastic sheets were placed on the ground. Faecal pellets were collected from the sheet approximately one week later, taking care to avoid pellets where urine had pooled (Möstl & Palme 2002). Faecal pellets were stored with ice-packs during transport (up to two hours) before being frozen at -80C.

4.7.2 Extraction of target compounds

A total of 18 samples were analysed; three replicates from each of six sites were processed. Analysis was based on the average of these three replicates.

Faecal samples were freeze-dried to remove water before being ground and homogenized (Bead Mill Homogenizer, BioSpec, USA). Each sample consisted of approximately 10mg of dried faeces was transferred to Eppendorf tubes with 600µl of methanol. Samples were homogenized with a vortex (Vortex Mixer; Alpha Laboratories, UK) and sonicated to further break down faecal matter. Each sample was then centrifuged at 13,000 rpm for 10 minutes. 500 µl of supernatant was transferred to a new Eppendorf with 500µl of 1% formic acid. Each sample was then pipetted on to an SLE+ isolute column (1ml, Biotage, Swden) attached to a vacuum pump and washed through with 2.5ml ethyl acetate twice as per the manufacturer's instructions. The filtered sample was collected in a 15ml falcon tube and dried under nitrogen gas in falcon tube before being stored at -80C.

4.7.3 Sample preparation for LC-MS QQQ analysis

Samples were re-suspended in 500µl of 50% methanol, 50% water, 0.1% formic acid spiked with umbelliferone. The umbelliferone served as an internal standard. The sample was then vortexed for 20 seconds and transferred into new Eppendorf tubes which were centrifuged for 5 minutes. The whole sample was then filtered into MS glass vials through a 0.45 µl filter tip.

4.7.4 Quantitative LC-MS QQQ analysis

Quantitative analysis of hormones was performed using an Agilent 6420B triple quadrupole (QQQ) mass spectrometer (Technologies, Palo Alto, USA) hyphenated to a 1200 series Rapid Resolution HPLC system. 10 µl of sample extract were loaded onto an Eclipse Plus C18 3.5 µm, 2.1 x 150 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA). For detection using positive ion mode, mobile phase A comprised of 100% LC-MS grade H2O, with 0.1% Formic Acid and mobile phase B was 100% Methanol (LC-MS grade) with 0.1% Formic Acid. The following gradient was used: 0 min – 40% B; 20 min – 100% B; 25 min – 100% B; 26 min – 40% B followed by 4 min re-equilibration time. The flow rate was 0.25 mL min^{-1} and the column temperature was held at 35 °C for the duration. The QQQ source conditions for electrospray ionisation were as follows: gas temperature 350 °C, drying gas flow rate of 11 l min-1, nebuliser pressure 35 psig, and capillary voltage 4 kV. All ions were scanned in positive ion mode and given a dwell time of 30 mseconds. The fragmentor voltage and collision energies had previously been optimised for each compound (Appendix 4.3). A 10uM mix containing each of these hormone standards was diluted by half each time to produce a concentration range from 10uM to 0.156uM. A blank

(extraction solvent spiked with umbelliferone) was run every 6 samples, and a 0.156 uM hormone mix every 12 samples as a quality control.

4.7.5 Mass spectrum data analysis

Data analysis was undertaken using Agilent Mass Hunter Quantitative analysis software for QQQ (Version B.07.01). Calibration curves were generated for each of the hormone standards (Appendix 4.4) which enabled the concentration of target compounds within samples to be ascertained. Concentrations were then standardised by sample weight. Precision (%Relative Standard Error) was also calculated (Appendix 4.5).

4.7.6 Road, habitat and weather data

In ArcGIS, I calculated the distance of each roost to the nearest A, B and minor road as well as the density of each type of road within core sustenance zones. I also calculated the size of the road-defined patch, the area bordered by A and B roads within which each roost was situated. Finally, I quantified the area of broadleaved woodland and built environments within both the core sustenance zone and the road-defined patch.

The size of the core sustenance zone varies by species and depends on the typical distance travelled by bats from the roost. *R. hipposideros* and *P. auritus* are thought to travel up to 2 and 3 kilometres from the roost respectively, which equates to a core sustenance zone of approximately 12 km^2 for *R. hipposideros* and 28 km^2 for *P. auritus*. In order to correct for the difference in the size of core sustenance zones

used by each species and between roosts in different sized road-defined patch areas, I used the percentage of the area occupied by broadleaved woodland or built environments rather than the actual area.

Weather data were provided by weather station records (accessed 2016); The average temperature, wind speed and the number of rainfall events were calculated from 24 hours before faecal sample collection began until the date that samples were removed; e.g. for samples collected between day 2 and 8, the average temperature between day 1 and 8 was calculated.

4.7.7 Statistical analysis

Correlations between environmental predictor variables and pre-treatment levels of target compound response variables were tested with both Pearson's correlation coefficient and Spearman's rank correlation.

Due to high levels of intercorrelation between target compounds, I conducted variable reduction (principle components analysis) on a larger dataset that included pre-treatment samples and post-treatment samples taken from the same roosts. All samples were included in the PCA in order that a direct comparison could be made between pre- and post-treatment samples as part of an experiment presented in section 4.9.

Principle component scores for each species and for both sexes within species were plotted against each other (e.g. PC 1 against PC 2 and PC 1 against PC 4) in order to determine whether the focal species differentiated from each other.

I then correlated the principle component scores of the pre-treatment samples against environmental predictor variables, again with both Pearson's correlation coefficient and Spearman's rank correlation.

I have chosen to not use a mnemonic name in place of, for example, "principle component 1" as is the convention. As an example, I could refer to a component that summarises mostly reproductive hormones as the "reproductive hormone component" to aid understanding and interpretation. However, I have chosen to use neutral terms because heightened levels of corticosteroid targets could indicate allostatic load or allostatic overload. In other words, it is not clear whether these components should be referred to as "stress components" or "allostatic load components".

4.8 Results of the correlative study

Eleven out of 15 target compounds were successfully extracted and identified from faecal samples (Table 4.5; for a complete list of target compounds including those not identified see Appendix 4.3). The position of each identified compound on the biosynthesis pathways is shown in Appendix 4.6.

Target compound	Action
Aldosterone	Corticosteroid
Cortisone	Corticosteroid
Hydrocortisone (cortisol)	Corticosteroid
11-deoxycorticosterone	Corticosteroid
Dihydrotestosterone	Reproductive hormone
Estriol	Reproductive hormone
Estrone	Reproductive hormone
17β-Estradiol	Reproductive hormone
Androsterone	Reproductive hormone
Pregnenolone	Precursor to both sex and stress hormones
17α-hydroxyprogesterone	Precursor to both sex and stress hormones
Testosterone	Unidentified
Progesterone	Unidentified
1-dehydrotestosterone	Unidentified
Corticosterone	Unidentified

Table 4.5 A list of target compounds including corticosteroids and reproductive hormones or their metabolites and precursors to both corticosteroids and reproductive hormones.

4.8.1 Correlations between environmental predictor variables and target compound response variables

Fourteen out of 187 correlations between environmental predictor variables (n=17) and endocrinological target compounds (n=11) were significant at the conventional level of statistical significance (Pearson's correlation, n=1; Spearman's rank correlation, n=6; both Spearman's and Pearson's, n=7; Table 4.9). After

Bonferroni correction (α = 0.05/187 = 0.0002), none of these correlations remained significant. Overall, there was no detectable effect of environmental variables on individual steroid levels.

Scatter plots of all correlations between environmental predictor variables and each of the eleven compounds are provided in Appendices 4.7-4.22)

4.8.2 Ordination of endocrinological target compounds - Principle component analysis

The levels of focal steroids were highly intercorrelated (Table 4.5). The strongest positive correlations were between compounds positioned closely on the steroid hormone biosynthesis pathway (Appendix 4.6). For example, cortisone is a metabolite of hydrocortisone (cortisol) whilst estrone and 17β-estradiol are closely related oestrogens. Androsterone, estriol and dihydrotestosterone share several precursors.

Principle component analysis produced four distinct components (Table 4.7) with Eigen values above 1 (Figure 4.9 and Table 4.8). Principle component 1 (PC 1) accounts for 25% of variance (Table 4.8) and summarises variation in three corticosteroids (aldosterone, cortisone, 11-deoxycorticosterone) and three reproductive hormones (dihydrotestosterone, estriol and androsterone; Table 4.7). All corticosteroids are negatively correlated with PC 1, i.e. decreasing as PC 1 increases and increasing as PC 1 decreases. All reproductive hormones are also positively correlated with PC 1, (Table 4.7). Reproductive hormones have stronger correlations with PC 1 than corticosteroids.

Principle component 2 (PC 2) accounts for 18% of variance (Table 4.8) and summarises variation in three corticosteroids (cortisone, hydrocortisone and 11-deoxycorticosterone) and one precursor to both corticosteroids and reproductive hormones (17α-hydroxyprogesterone; Table 4.7). Two corticosteroids (cortisone and hydrocortisone) and one precursor to both corticosteroids and reproductive hormones (17α-hydroxyprogesterone) are positively correlated to PC 2, increasing as PC 2 increases and decreasing as PC 2 decreases. One corticosteroid (11-dexocycorticosterone, a precursor to aldosterone) is negatively correlated to PC 2, decreasing as PC 2 increases and increasing as PC 2 decreases. Cortisone and hydrocortisone have stronger correlations with PC 2 than 17α-hydroxyprogesterone.

Principle component 3 (PC 3) accounts for 11% of variance (Table 4.8) and summarises two reproductive hormones (estrone, 17β-estradiol) which are both positively correlated with PC 3, increasing as PC 3 increases and decreasing as PC 3 decreases (Table 4.7). One precursor to both corticosteroids and reproductive hormones (17α-hydroxyprogesterone) is negatively correlated with PC 3, increasing as PC 3 decreases and decreasing as PC 3 increases. Corticosteroids have stronger correlations with PC 3 than the precursor to both corticosteroids and reproductive hormones.

Principle component 4 (PC 4) accounts for 11% of variance (Table 4.8) and is composed of one corticosteroid (aldosterone), a precursor to a corticosteroid (11-deoxycorticosterone) and two precursors to both corticosteroids and reproductive hormones (pregnenalone and 17α-hydroxyprogesterone; Table 4.7). One corticosteroid (11-deoxycorticosterone) is positively correlated with PC 4, increasing as PC 4 increases and decreasing as PC 4 decreases. One corticosteroid (aldosterone) is negatively correlated with PC 4, decreasing as PC 4 increases and

increasing as PC 4 decreases. Both precursors to both corticosteroids and reproductive compounds are positively correlated with PC 4, increasing as PC 4 increases and decreasing as PC 4 decreases.

	Aldosterone		Androsterone		Cortisone		Dihydrotestosterone		Estriol		Estrone		Hydrocortisone		Pregnenolone		11-deoxycorticosterone		17β-Estradiol	
Aldosterone																				
Androsterone	-.235	-.216																		
Cortisone	-.066	.142	-.342	-.671**																
Dihydrotestosterone	-.280	-.472*	.909**	.582**	-.455*	-.516*														
Estriol	-.288	-.177	.619**	.615**	-.462*	-.418	.760**	.432*												
Estrone	-.241	-.365	.211	.278	-.015	.026	.272	.215	-.048	-.217										
Hydrocortisone	.035	.451*	-.220	-.413	786**	.733**	-.262	-.432*	-.318	-.361	.212	.171								
Pregnenolone	-.201	.029	-.211	.147	.007	.027	-.139	-.033	.015	.243	-.200	-.130	.151	-.099						
11-deoxycorticosterone	-.130	-.009	-.140	-.426*	-.046	.253	-.213	-.168	-.060	-.067	-.359	-.150	-.182	.161	.445*	.340				
17α-hydroxyprogesterone	-.551**	-.237	-.121	.126	.306	.258	-.033	.167	.103	.219	-.052	-.036	.239	.083	.255	.295	-.079	-.217		
17β-Estradiol	-.119	-.329	.017	.024	.030	.072	-0.72	-.117	-.274	-.341	.738**	.709**	.111	.048	-.321	-.284	-.316	-.273	-.118	-.190

Table 4.6 The results of intercorrelations between target compounds. For each intercorrelation, the result of Pearson's correlation is reported on the left side of the column and the result of Spearman's correlation is reported on the right. Results highlighted in light pink squares are significant (<0.05) and those highlighted in dark pink are highly significant (<0.01).

Target compound	Role	PC 1	PC 2	PC 3	PC 4
		(25.10)	(18.07)	(14.99)	(11.08)
Aldosterone	Corticosteroid	-.385			-.668
Cortisone		-.337	.832		
Hydrocortisone (cortisol)			.892		
11-deoxycorticosterone		-.361	-.430		.524
Dihydrotestosterone	Reproductive hormone	.828			
Estriol		.471			
Estrone				.907	
17β-Estradiol				.859	
Androsterone		.868			
Pregnenolone	Precursor				.682
17α-hydroxyprogesterone			.437	-.480	.466

Table 4.7 Rotated component loadings for each principle component. The percentage variance that each component accounts for is provided in brackets.

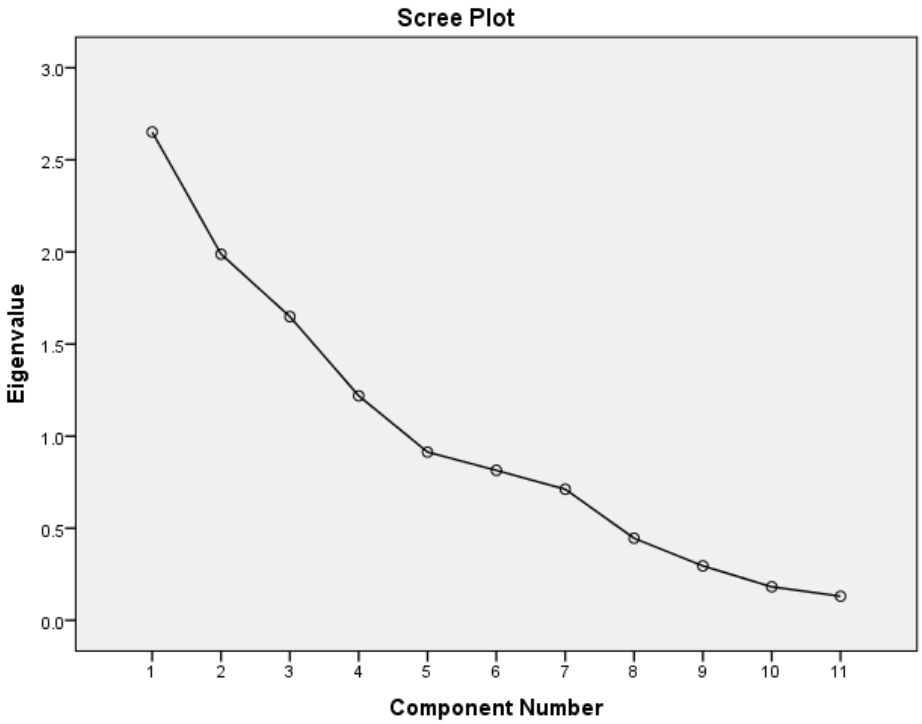


Figure 4.9 Scree plot of Eigen values for each principle component.

Component	Total	% of variance	Cumulative variance
1	2.65	25.10	24.10
2	1.98	18.07	42.18
3	1.64	14.99	57.17
4	1.21	11.08	68.25

Table 4.8 The Eigen value of each principle component as well as the proportion of variance accounted for by each principle component and cumulative variance.

4.8.3 Ordination: differentiation of groups by their endocrinological profiles using PCA scores

As PCA was performed on a larger dataset including pre- treatment and post-treatment samples, all samples are included in the ordinations. Ordination plots reveal that *P. auritus* and *R. hipposideros* scores differentiate for PC 1 (Figure 4.10 a). *P. auritus* tends to have higher scores for PC 1 than *R. hipposideros* with some overlap at central scores. There is a great deal of overlap between species in their scores for PC 2 (Figure 4.10 a), PC3 and PC 4 (Figure 4.10 b), although *P. auritus* tends to have a wider range of scores for PC 2 and *R. hipposideros* has a wider range of scores for PC 4.

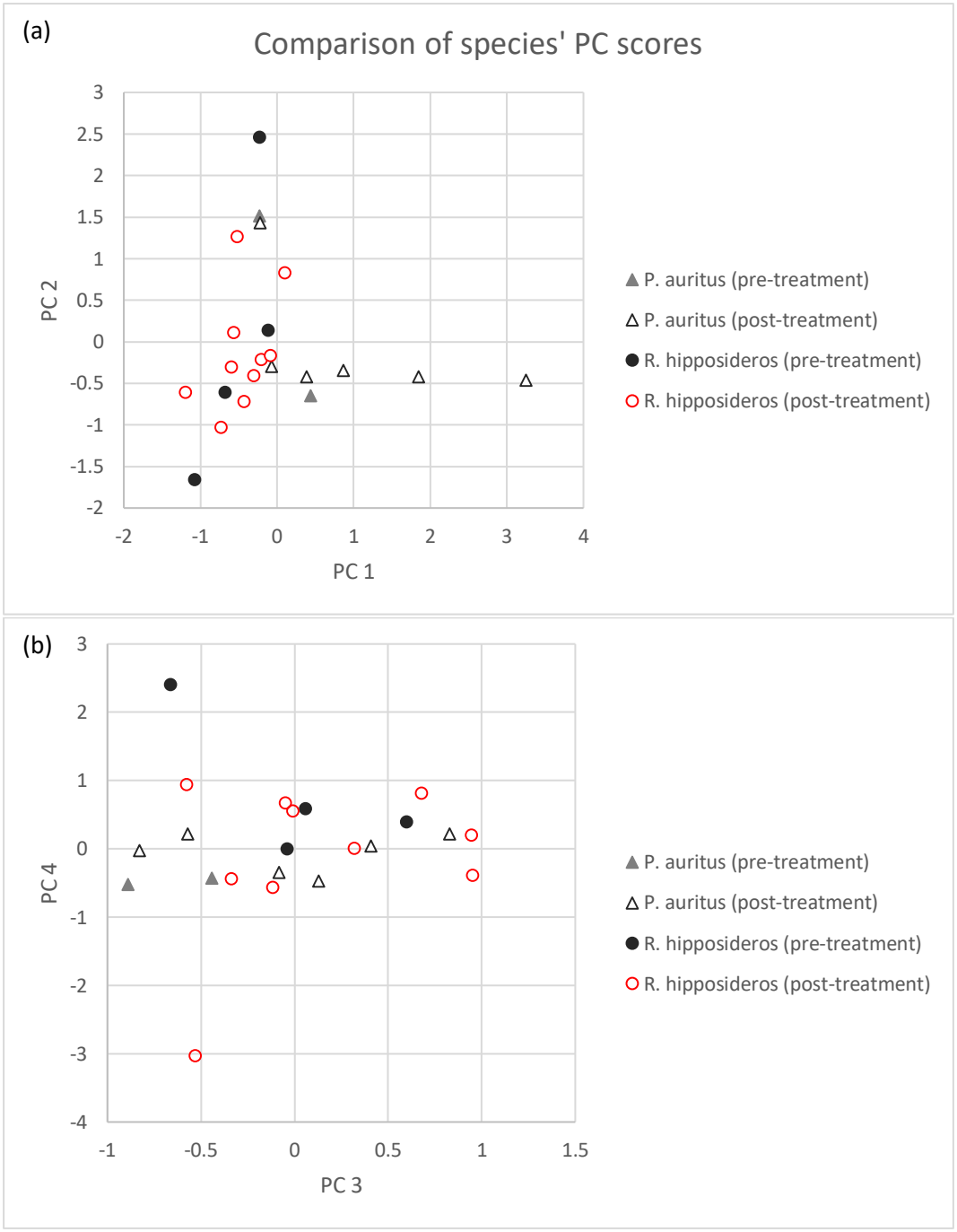
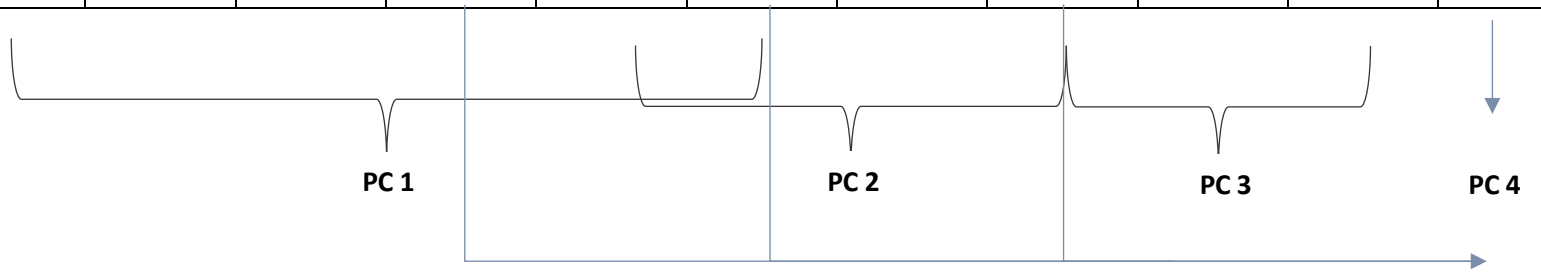


Figure 4.10 Ordination plots of principle components for PC 1 against PC 2 (a) and PC 3 against PC 4 (b). P. auritus pre-treatment n=2, P. auritus post-treatment n=6, R. hipposideros pre-treatment n=4, R. hipposideros post-treatment n=10.

4.8.4 Correlations between environmental predictor variables and principal component response variables

Seven out of 68 correlations between environmental predictor variables and principal component response variables were significant (Pearson's coefficient correlation, n=1; both Pearson's coefficient correlations and Spearman's rank correlation, n=6; Table 4.9). Following Bonferroni correction ($\alpha = 0.05/68 = 0.0007$) none of these correlations remained significant. Overall, there was no detectable effect of environmental variables on endocrinological profiles.

	Andro-sterone	Dihydro-testosterone	Estriol	Aldosterone	Cortisone	11-Deoxycorticosterone	Hydro-cortisone	17α-Hydroxyprogesterone	17β-Estradiol	Estrone	Pregnen-olone
Distance to nearest road (m)											
All	-0.314	-0.217	-0.602 S*	-0.409	-0.294	0.158	-0.341	-0.681	0.444	0.507	0.027
A	0.137	-0.835 S*	0.137	0.9 P* S*	0.842	-0.280	0.897 P* S*	0.506	-0.473	-0.355	-0.071
B	0.088	0.241	0.122	-0.417	-0.632		-0.835	-0.184	0.491	0.4	-0.23
Minor	-0.319	-0.225	-0.616	-0.45	-0.298	0.138	-0.362	-0.654	0.494	0.552	0.015
Road density (m/m²)											
All	-0.579	-0.733	-0.341	-0.631	0.08	0.751	0.186	0.121	0.067	-0.056	0.934 P** S**
A	-0.497	-0.449	-0.458	-0.89 P* S*	-0.258	0.333	-0.495	-0.024	0.617	0.515	0.312
B	0.477	0.032	0.141	0.241	0.481	-0.542 S*	0.827 P*	0.665	0.191	0.151	0.03
Minor	-0.576	-0.694	-0.297	-0.528	0.075	0.797	0.19	0.052	-0.069	-0.175	0.925 P** S**
Core Sustenance Zone attributes											
Broadleaved woodland (%)	-0.413	-0.528	-0.459	-0.577	-0.246	0.678	-0.029	-0.422	0.256	0.165	0.801
Built environment (%)	-0.359	-0.289	-0.21	-0.612	0.032	0.007	-0.338	0.408	0.420	0.358	-0.028
Patch attributes											
Patch size (km²)	0.793	0.525	0.656 S*	0.703	0.091	-0.379	0.52	0.296	-0.306	-0.369	-0.052
Broadleaved woodland (%)	0.307	-0.163	-0.042	-0.137	0.430	-0.471	0.691	0.760	0.497	0.406	0.155
Built environment (%)	0.725	-0.116	0.522	-0.058	0.087	-0.463	0.257	0.696	0.203	0.203	0.290
Weather conditions											
Average temperature (C°)	0.666	0.586	0.475	0.375	0.033	-0.756 S*	0.019	0.451	0.152	0.127	-0.614
Minimum temperature (C°)	0.85 P* S**	0.598	0.583	0.58	-0.268	-0.471	0.049	0.272	0.24	0.08	-0.12
Average wind speed	0.539 S**	0.103	0.235	0.322	0.321	-0.369	0.781	0.46	0.062	-0.003	0.178
Average rain fall	0.313	-0.172	0.003	-0.169	0.125	-0.071	0.594	0.384	0.389	0.231	0.549



	PC 1		PC 2	PC 3	PC 4	
Distance to nearest road (m)						
All	-0.363		-0.246	0.253	-0.214	
A	0.235		0.748	-0.394	-0.118	
B	0.281		-0.746	0.422	0.055	
Minor	-0.35		-0.248	0.309	-0.215	
Road density (m/m^2)						
All	-0.872	P* S*	-0.138	0.16	0.909	P* S**
A	-0.466		-0.388	0.657	0.407	
B	0.458		0.760	0.145	-0.179	
Minor	-0.89	P* S*	-0.161	0.018	0.903	P* S**
Core Sustenance Zone attributes						
Broadleaved woodland (%)	-0.815	P* S*	-0.297	0.152	0.549	
Built environment (%)	-0.086		-0.129	0.586	0.259	
Road defined patch attributes						
Patch size (km^2)	0.592		0.349	-0.342	-0.18	
Broadleaved woodland (%)	-0.901	P*	-0.662	-0.31	0.727	
Built environment (%)	-0.564		-0.246	-0.461	0.577	
Weather conditions						
Average temperature (C°)	0.899	P* S**	0.26	0.183	-0.506	
Minimum temperature (C°)	0.695		0.063	0.173	-0.201	
Average wind speed	0.353		0.583	0.043	-0.061	
Average rain fall	-0.015		0.341	0.373	0.285	

Table 4.9 Pearson's correlation coefficients between environmental variables and target compounds (a) and principle components (b). Significance is reported for both Pearson's (P) and Spearman's (S) correlations. Light pink indicates significance at the 0.05 level and dark pink indicates significance at the 0.01 level.

4.8.5 Road density

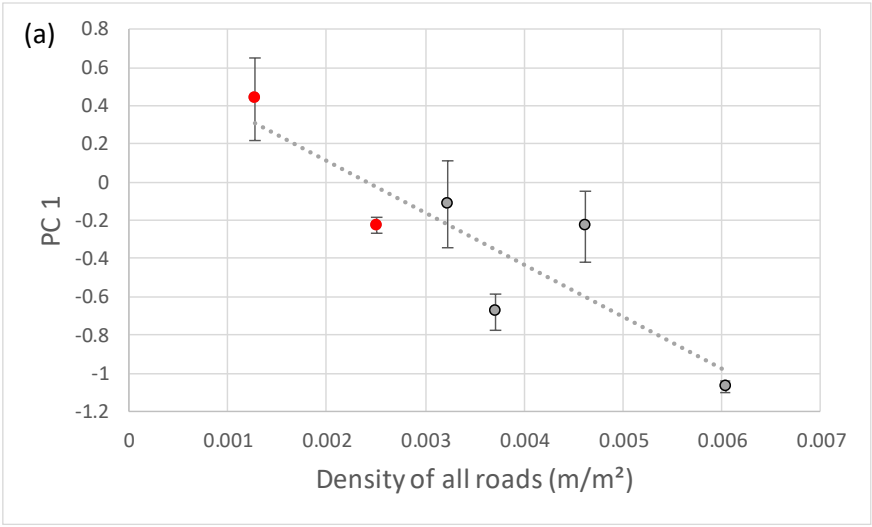
The density of all roads and minor roads negatively correlated with PC 1 (Figure 4.11 a and b) and positively correlated with PC 4 (Figure 4.11 f and g). Between them, PC 1 and PC 4, summarised most of the target stress hormone-related compounds, with the exception of hydrocortisone (cortisol), estrone and 17β-estradiol (Table 4.10).

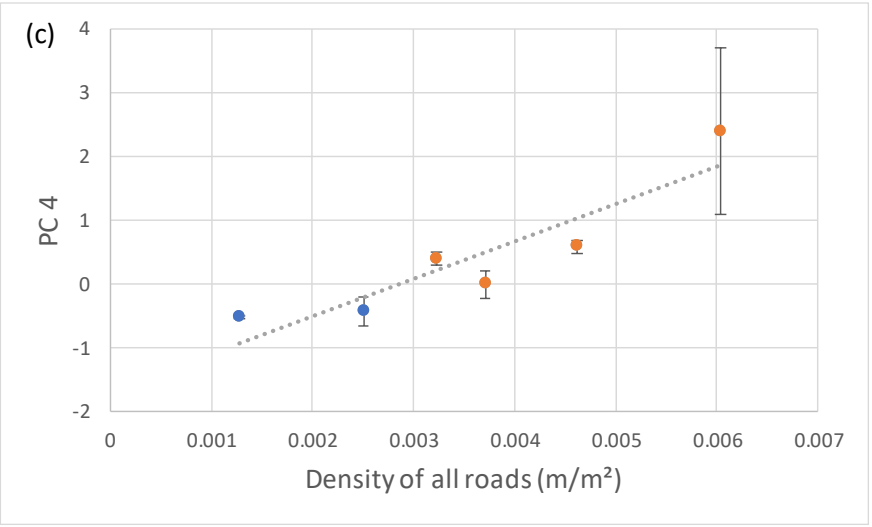
4.8.6 Habitat quality

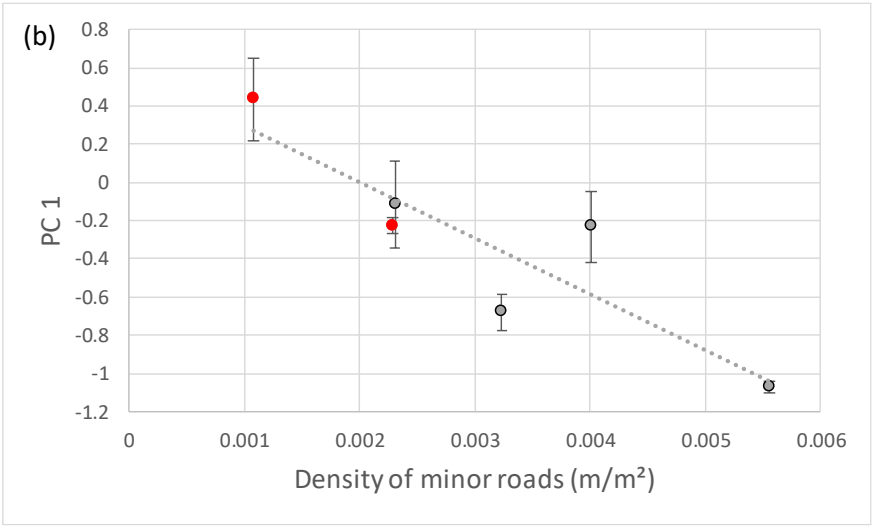
Broadleaved woodland as a percentage of both CSZs and road-defined patches negatively correlated with PC 1 (Figure 4.11 c and d).

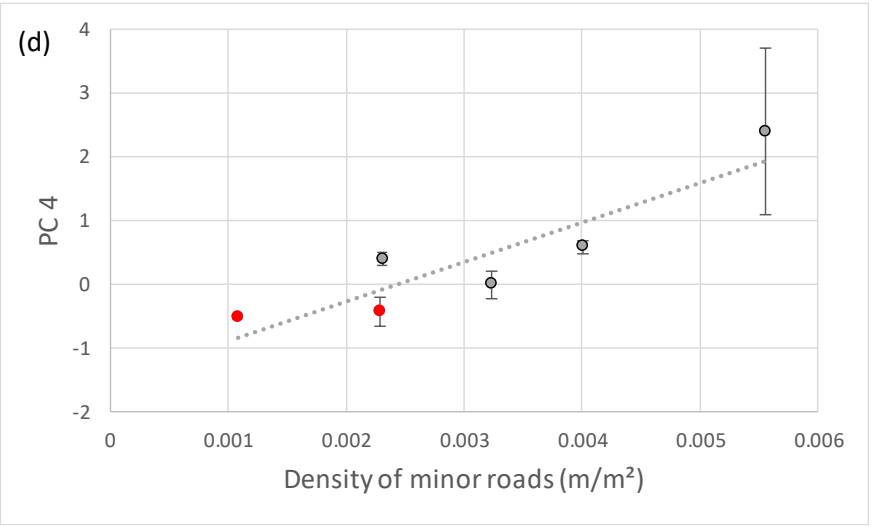
4.8.7 Weather conditions

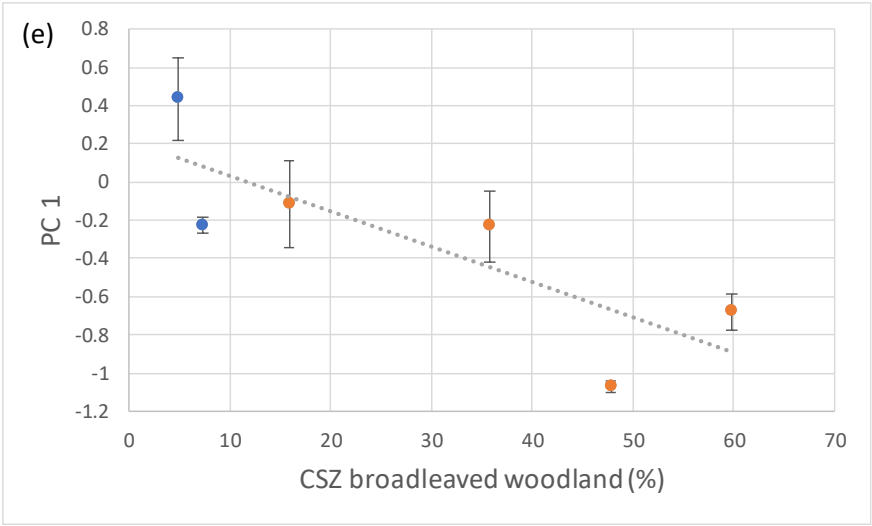
Average temperature positively correlated with PC 1 (Figure 4.11 e). Scatter plots of all correlations between environmental predictor variables and principle components are provided in Appendices 4.22 – 4.38.

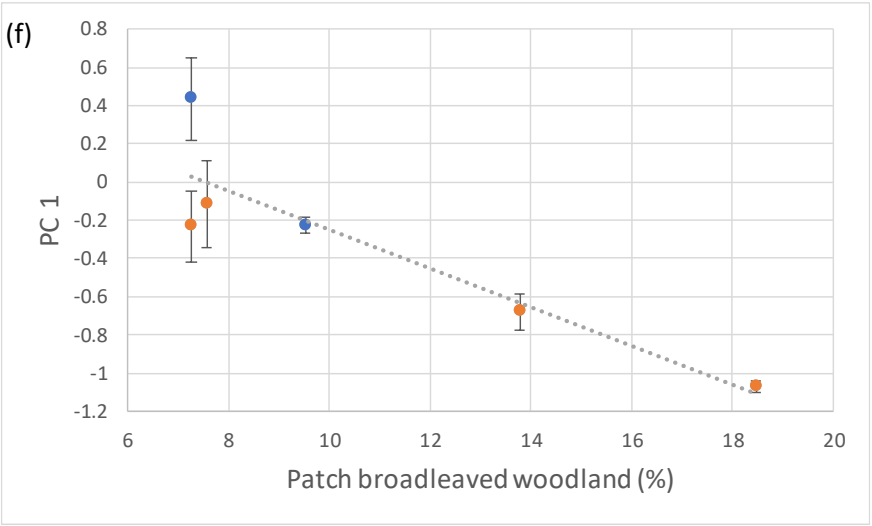

(a)
PC 1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
0
0.001
0.002
0.003
0.004
0.005
0.006
0.007
Density of all roads (m/m²)


(c)
PC 4
4
3
2
1
0
-1
-2
0
0.001
0.002
0.003
0.004
0.005
0.006
0.007
Density of all roads (m/m²)


(b)
PC 1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
0
0.001
0.002
0.003
0.004
0.005
0.006
Density of minor roads (m/m²)


(d)
PC 4
4
3
2
1
0
-1
-2
0
0.001
0.002
0.003
0.004
0.005
0.006
Density of minor roads (m/m²)


(e)
PC 1
CSZ broadleaved woodland (%)


(f)
PC 1
Patch broadleaved woodland (%)

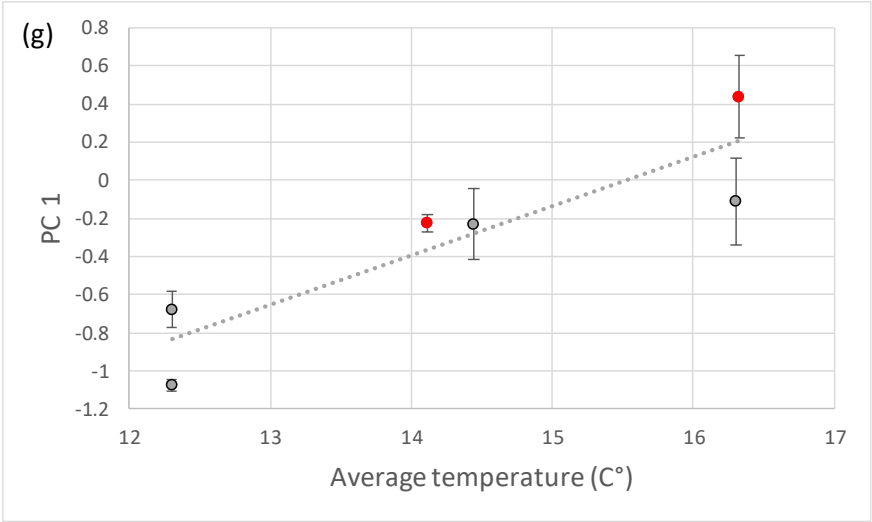

(g)
PC 1
Average temperature (C°)

Figure 4.11 Scatter plots of principle components and environmental predictor variables; PC 1 and the density of all roads (a), PC 1 and the density of minor roads (b), PC 4 and the density of all roads (c) and PC 4 and the density of minor roads (d), PC 1 and the percentage of broadleaved woodland within the CSZ (e), PC 1 and the percentage of broadleaved woodland within road-defined patches (f), PC 1 and average temperature (g). Only those correlations that are significant are presented here, all correlations including those that were not significant are provided in Appendix 4.23-4.39. Closed red circles = *P. auritus* open circles = *R. hipposideros*.

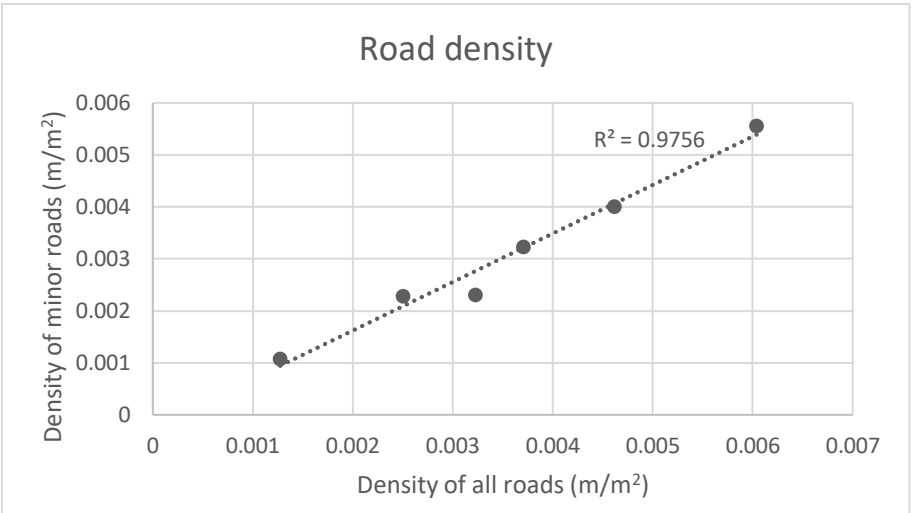
Target compound	**Role**	**PC 1**	**PC 4**
		(25.10)	(11.08)
Aldosterone	Corticosteroid	+	-
Cortisone		+	
11-deoxycorticosterone		+	+
Dihydrotestosterone	Reproductive hormone	-	
Estriol		-	
Androsterone		-	
Pregnenolone	Precursor		+
17α-hydroxyprogesterone			+
Hydrocortisone (cortisol)	Corticosteroid		
Estrone	Reproductive hormone		
17β-Estradiol	Reproductive hormone		

Table 4.10 The direction of the correlation between road density (all and minor) and each compound within PC 1 and PC 4; positive (+) and negative (-). The percentage variance that each component accounts for is shown in brackets.

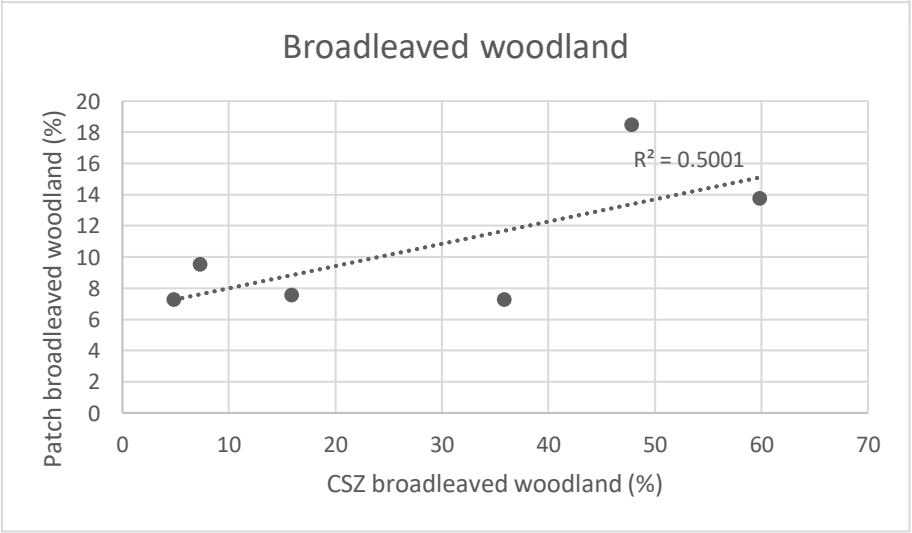
4.8.8 Intercorrelations between environmental variables

The density of all roads and minor roads were highly correlated suggesting that most roads within a CSZ were minor roads (r = 0.97; Figure 4.12 a). The percentage of broadleaved woodland within CSZs and road defined patches were moderately correlated (r = 0.5; Figure 4.12 b) and the percentage of broadleaved woodland within CSZs and the density of minor roads were moderately correlated (r = 0.56; Figure 4.12 c).

(a)



(b)



(c)

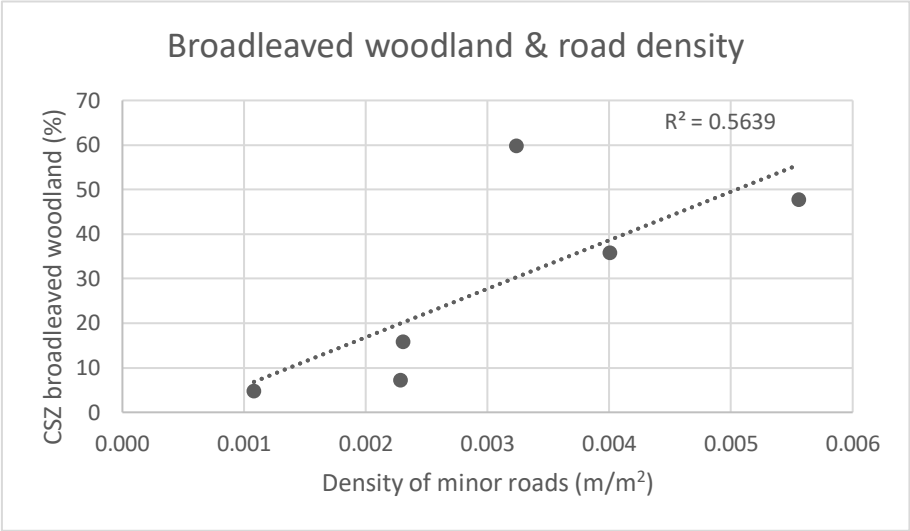


Figure 4.12 Scatter plots of intercorrelations between environmental predictor variables including the density of all roads and minor roads (a), the percentage of broadleaved woodland within CSZs and road-defined patches (b), and the density of minor roads and the percentage of broadleaved woodland within CSZs (c).

4.9 Discussion

Faecal corticosteroids and reproductive hormones correlated with two predictor variables: road density and average temperature, which begins to suggest that faecal corticosteroid profiles may reveal a stress signature in bats. As expected faecal corticosteroids and reproductive hormones are negatively correlated with each other; where faecal corticosteroids increase, reproductive hormones decrease, which is consistent with biological understanding and therefore begins to validate the presence of reliable patterns of the endocrinological target compounds identified in faecal samples taken from bats.

4.9.1 Road density

Apparently, faecal corticosteroids within roosts are influenced by road density, primarily the density of minor roads, within the core sustenance zone. The density of all roads and minor roads were highly correlated with each other which suggests that most roads within CSZs were minor roads.

Overall, the density of minor roads is positively correlated with most (four of six; Table 4.10) corticosteroids summarised in PC 1 and PC 4, whilst the percentage of broadleaved woodland within the CSZ (and road-defined patch) is negatively correlated with PC 1. This suggests that corticosteroids increase as road-density increases, but remarkably, corticosteroids also increase as the proportion of broadleaved woodland within the vicinity of a roost increases; i.e. more woodland seems to have a negative impact on the endocrinological profiles.

A potential explanation for this peculiar result is that the percentage of broadleaved woodland (within the CSZ or road-defined patch) was positively correlated with road density (all and minor). This may be due to the fact that roads in rural areas, especially minor roads, are often bordered by trees. It is possible therefore, that the percentage of broadleaved woodland within the CSZ is a proxy for road density within the CSZ.

These results not only indicate corticosteroids increase with increasing minor road density, but that the increased availability of broadleaved woodland associated with a greater density of minor roads does not mitigate or counteract the impact of roads.

Taken together, these patterns suggest that roadside habitats are less valuable foraging space than edge habitats away from the road, either because bats avoid the

open spaces created by roads or because passing traffic interferes with their ability to locate insect prey (Schaub et al. 2008; Siemers & Schaub 2011). As a result, road density has an effect on glucocorticoid levels as a result of it influence on food availability (E_G) and the energy invested in foraging (E_I). Alternatively, roadside habitats are useful foraging spaces and have no effect on foraging opportunities, food availability or foraging effort, and are used as frequently as edge habitats away from the road. However, bats foraging on or close to the road are more likely to be exposed to traffic which triggers a stress response (E_O).

We might infer, if spatial variation in road density can be used as the basis of a space-for-time substitution (Pickett, 1989), that building additional roads could increase corticosteroids levels and therefore allostatic load at nearby roosts. An important point to reiterate is that glucocorticoids may positively correlate with increasing road density but are not necessarily an indication of allostatic overload (stress). However, by increasing allostatic load, roads could render bats more vulnerable to allostatic overload in the event of additional stimuli or perturbances. If levels of stress are demographically significant, it is therefore advisable that during the planning stage of proposed new roads, surveys should be undertaken to ensure that maternity roosts are not present within 3-4 km of the planned route which would ensure that the new road does not fall within the CSZ of maternity roosts. Further work is required to establish the demographic relevance of variation in endocrine profiles.

4.9.2 Temperature

PC 1 was positively correlated with average temperature. As PC 1 increases with temperature, faecal corticosteroids decrease whilst reproductive hormones

increase. The decrease of corticosteroids in PC 1 with increasing temperature could be as a result of bats foraging for longer or their prey being more active in warmer temperatures.

Endocrinological profiles in faecal samples are doubtless susceptible to microbial degradation to some extent. If this was a substantive influence, it is expected that endocrinological profiles would be detectably associated with environmental temperature. The positive correlation between temperature and PC 1 may indicate that temperature influenced the yield of corticosteroids post-excretion, possibly through microbial action (Millspaugh & Washburn 2004, Sheriff et al. 2011). However, steady room temperature (22°C), high temperature (38°C) and alternating room temperature and high temperature had no affect on Faecal corticosteroid metabolite (FCM) yields from white-tailed deer faeces, whereas alternating freezing and room temperature increased FCM yields (Washburn and Millspaugh 2002). All maternity roosts were located in attics of occupied houses and were above room temperature at 10pm. It is possible that temperatures dropped during the night, but it is very unlikely to have dropped below zero.

Further, corticosteroids in PC 1 are negatively correlated with temperature whilst reproductive hormones are positively correlated. Individually, most compounds except 11- deoxycorticosterone and pregnenalone are positively (but non-significantly) correlated with temperature. This suggests that correlations between PC 1, and individual compounds, with temperature are not the effect of temperature on microbial activity as we might expect microbial activity to degrade all compounds.

4.9.3 Comments and caveats

I found a strong correlation between PC 1 and minor road density. The interpretation of these results presented here is at present tentative. These results could be artefactual owing to the fact that two different species were included in the analysis. Ordination reveals that the two focal species differentiate with regards to their endocrinological profiles. Specifically, *P. auritus* tends to exhibit higher PC 1 scores than *R. hipposideros* when considering both pre- and post-treatment samples. The significant correlations, therefore, between PC 1 and road density (all roads and minor) and the percentage of broadleaved woodland (within the CSZ and road-defined patch) are likely dependent on *P. auritus'* high PC 1 scores. However, I argue that there are two reasons to believe that these results are indicative of a real biological effect of roads on the endocrinological response of bats.

First, correlations between PC 1 and road density and PC 1 and broadleaved woodland remain moderate (r= 0.5 for both cases; Appendix 4.40) but are no longer significant after excluding *P. auritus*. Second, there appears to be less differentiation between the two species when we consider only the pre-treatment scores, which were correlated with environmental variables; PC 1 scores for both species are close to zero and overlap somewhat; *P. auritus* scores -0.22 and 0.4 whilst *R. hipposideros* scores between -1.07 and -0.11. Further research is needed to confirm whether these species do indeed have distinctly different pre-treatment scores for PC 1

Whilst PC 4 scores for *P. auritus* fall within the range of *R. hipposideros* when we consider all samples, *P. auritus* tends to exhibit lower PC 4 scores than *R. hipposideros* when we consider only pre-treatment samples. Significant correlations

between PC 4 and road density (all and minor) were driven by the low scores of *P. auritus* which strongly implies that this particular correlation could be an artefact.

In summary, there are reasons to doubt the interpretation of the results presented here, especially with regards to the influence of minor road density on the endocrinological response summarised in PC 1. But, it may be possible to test fully the reliability of these results by sampling an additional two *R. hipposideros* roosts.

4.9.4 Suggestions for future research.

In order to determine whether the correlation between corticosteroid levels and road density is indicative of chronic stress in *R. hipposideros* and *P. auritus*, future studies should also correlate corticosteroid levels with measures related to survival and fitness. For example, traffic density was associated with lower corticosterone in amphibians and also smaller body size which could be indicative of exhaustion (Cayuela.et al. 2017). Corticosteroid levels in female kestrels was negatively correlated with proximity to large, busy roads and positively correlated with the probability of nest abandonment (Strasser et al. 2013).

In bats, one suitable measure might be a comparison of average emergence counts (the number of bats observed exiting the roost) at the beginning and end of the maternity season as an indicator of breeding success or juvenile fledging success. During the maternity season, invasive measures such as body weight or condition are not suitable as handling bats will cause a stress response that could be detrimental to gestating bats and potentially cause the pregnancy to be terminated.

The results presented here suggest that even minor roads have an influence on corticosteroid levels at nearby roosts. In order to determine whether major roads

have an impact on corticosteroid levels at nearby roosts, future research should compare corticosteroids at roosts within CSZs with varying densities of major roads; i.e. corticosteroid levels should be correlated with the density of major roads. In order to determine whether major roads have a greater influence on stress than minor roads, the average PC scores of roosts within CSZs with a high density of major roads could be compared with average scores at roosts within CSZs with a high density of minor roads.

We might expect that minor roads have less of an impact on corticosteroid levels than major roads as minor roads tend to have less traffic, are less likely to be artificially lit and are less likely to act as a barrier to movement and are therefore less likely to restrict access to resources. On the other hand, although minor roads tend to have lower levels of traffic, it is possible that infrequent exposure to vehicles prevents bats from acclimatising to traffic.

I examined corticosteroid levels at maternity roosts. Due to the heightened energetic requirements of females nursing young, it is possible that individuals at maternity roosts are more sensitive to the impacts of roads on habitat quality and to the availability of suitable foraging habitat in the vicinity of roosts. Future research should compare the impact of roads and habitat quality on corticosteroid levels at day roosts outside of breeding season and at hibernation roosts.

In order to determine whether the *P. auritus* and *R. hipposideros* have different pre-treatment PC scores, and therefore how reliable the correlations between PC scores for both species and environmental variables are, more roosts of both species should be sampled. If the two species have significantly different pre-treatment PC

scores, separate correlations between PC scores and environmental variables would be more appropriate.

Broadleaved woodland and road density are correlated with each other. Consequently, variable reduction in the form of principle component analysis would be an appropriate additional analysis. PC scores for environmental conditions could be correlated against PC scores for target compounds.

4.10 An experimental study: The impact of lighting on glucocorticoids

4.10.1 Introduction to the question

Artificial lighting can have a wide range of impacts on the biology and ecology of wild animals. Some species avoid artificial lighting; their ranges and foraging spaces restricted to unlit locations (Longcore & Rich 2004). The downstream effects of avoidance include restricted availability of resources and reduced connectivity between populations (Gaston & Bennie 2014). Some species are attracted to artificial lighting (e.g. amphibians and arthropods), which could make them vulnerable to predation (Longcore & Rich 2004). Turtles and birds can be disorientated by artificial lighting; hatchlings have been observed moving towards artificial lights rather than the ocean leading to high rates or mortality (Salmon et al. 1995). Birds can become trapped in “light-islands” surrounding tall buildings during migration (Ogden 1996). Artificial lighting can also impact the physiology of organisms including the immune system, metabolism and the endocrine system (Navara & Nelson 2007).

Relatively little research has been conducted on the impact of artificial lighting on endocrinological responses of wild terrestrial vertebrates. However, artificial lighting could potentially impact reproductive hormones in wild animals. Ambient light levels and the length of the photoperiod are cues for reproductive behaviours and processes. This adaptation ensures that young are born during optimal conditions, i.e. when resources are available and the temperature is mild. Exposure to persistent artificial lighting is associated with disruption to these processes. For example, *Tardus merula* individuals exposed to artificial lighting were found to develop their reproductive system up to a month earlier, and to moult earlier, than the control group (Dominoni et al. 2013). Similarly, by masking the length of the photoperiod, artificial lighting causes a delay in the onset of birth in Tamar wallabies (Robert et al. 2015).

Artificial lighting has been shown to have an impact on stress hormones in birds. Lighting is associated with increased stress levels in free-living great tits (Parus major), where corticosterone levels were found to be higher in transects lit by white light compared to unlit transects (Ouyang et al. 2015). In free-living European blackbirds (Tardus merula) corticosteroid levels were positively correlated with the density of lamps within 50m of the nest (Russ et al. 2015). In captive male mice, constant artificial lighting for six weeks was associated with reduced corticosterone levels possibly as a result of downregulation in response to a chronic stressor (Fonken et al. 2009).

There are good reasons therefore to expect that artificial lighting could have an impact on the stress response in bats. Several species of bat are known to avoid artificial lighting, including *R. hipposideros*. Because some species avoid artificial lighting, lights bordering roads contribute to the barrier effect of roads for bats (Chapter 2). Avoidance behaviour in response to artificial lighting could be mediated by a stress

response. It is clear that a potential light-stress relationship deserves further investigation in bats.

To examine whether artificial lighting is associated with an increase in corticosteroids in bats, I conducted an experimental study. Comparisons were made between pre-treatment corticosteroid levels and two post-treatment samples at six maternity roosts. An increase in glucocorticoids following initial exposure to artificial lighting could be indicative of an emergency stress response. A decrease in corticosteroids following exposure to artificial lighting could be indicative of downregulation of the HPA axis as a result of chronic stress.

4.11 Methods

4.11.1 Collection of faecal samples

Six maternity roosts (*Rhinolophus hipposideros* n = 4; *Plecotus auritus* n = 2) were visited between June and September 2015 (for exact dates see Appendix 4.41). The majority of individuals in maternity roosts were pregnant females or females rearing young. Any males in the roost were likely to have been juveniles.

Roosts were entered after dark at approximately 10pm when most bats had left the roost. Plastic sheets were placed on the ground (visit 1) and left in position for the duration of the study. Faecal pellets were collected from the sheet one a week (visits 2-5, Table 4.11), taking care to avoid pellets where urine had pooled, and stored with ice-packs during transport (up to two hours) before being frozen at -80C. Pre-treatment samples were collected on visit 2, and post-treatment samples were collected on visits 3-5 (Table 4.11).

Lights were positioned on tripods at a height of about 2m from the ground, on either side of a commuting route between the roost and potential foraging areas. The lights were angled facing each other so that the light fell across the flight path. The lights were situated at a minimum of 20 metres from the roost (visit 2). Likely commuting routes were identified by speaking to roost owners and observing emergence behaviour at sunset on visits 1 and 2. Commuting routes tended to follow linear features, such as treelines and hedges, and were directed towards nearby broadleaved woodland. The lights used were security lights (Safego, CREE LED spot light, colour temperature 6000K~6500K, 72W, 2160 lumen).

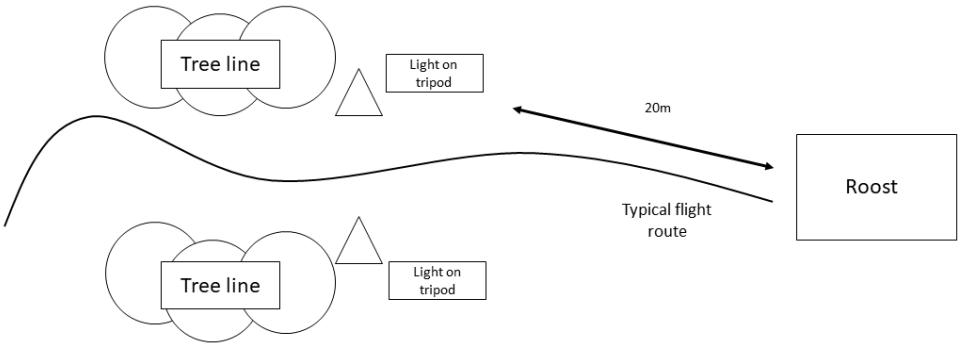


Figure 4.13 The typical position of lights relative to the focal roost. Lights were positioned on tripods either side of a known flight path, about 20m from the roost.

Visit	Treatment	Activity
1		Place plastic sheeting
2	*No lighting*	Pre-treatment samples collected, lights positioned.
3	*Lighting for previous 7 days*	First post-treatment samples collected
4	*Lighting for previous 14 days*	Second post-treatment samples collected, lights dismantled
5	*No lighting for previous 7 days*	Third post-treatment samples collected

Table 4.11 The treatment protocol used for this experiment.

4.11.2 Extraction and quantitative analysis of endocrinological target compounds

The extraction and quantitative analysis of target compounds was performed as described above (sections 4.7.2, 4.7.3, 4.7.4 and 4.7.5). A total of 66 samples were analysed; three replicates for each treatment at each roost. Analysis is based on the average of these three replicates, i.e. 22 averaged samples. All reported concentrations are based on dried weight of samples.

4.11.3 Statistical analysis

Principle component analysis was conducted as described above (in 4.7.7) and produced four components (Table 4.7).

The number of samples per treatment for each species and both species combined is shown in Table 4.12. Post-treatment 1 and 2 samples (samples collected at the end of 7 and 14 days exposure to artificial lighting, respectively) were combined for analysis (Table 4.13).

Paired samples t-tests were performed on pairs of treatment categories due to unequal sample sizes across treatment groups. Due to small sample sizes, paired t-tests were performed on both species combined and on *R. hipposideros* but not *P. auritus* (Table 4.14).

Treatment	Variable name	*P. auritus*	*R. hipposideros*	*Both species*
No lighting	*Pre-treatment*	*2*	*4*	*6*
Lighting for previous 7 days	*Post-treatment 1*	*3*	*4*	*7*
Lighting for previous 14 days	*Post-treatment 2*	*2*	*3*	*5*
No lighting for previous 7 days	*Post-treatment 3*	*1*	*3*	*4*

Table 4.12 The number of samples for each treatment for each species and both species combined.

Treatment	Variable name	*P. auritus*	*R. hipposideros*	*Both species*
No lighting	*Pre-treatment*	*2*	*4*	*6*
Lighting for previous 7-14 days	*Post-treatment 1 and 2*	*5*	*7*	*12*
No lighting for previous 7 days	*Post-treatment 3*	*1*	*3*	*4*

Table 4.13 The number of samples for each species and both species combined.

Variable 1	Variable 2	*P. auritus*	*R. hipposideros*	*Both species*
Pre-treatment	*Post-treatment 1 and 2*	*2*	*4*	*6*
Post-treatment 1 and 2	*Post-treatment 3*	*1*	*3*	*4*

Pre-treatment	*Post-treatment 3*	*1*	*3*	*4*

Table 4.14 The number of samples in each paired t-test. It was not possible to conduct t-tests for *P. auritu*s due to insufficient samples.

4.12 Results

4.12.1 Testing the effect of additional lighting

Additional lighting did not affect levels of individual hormones in bats. There were no significant differences between any two treatment categories for either samples taken from *R. hipposideros* roosts or for both species combined (Table 4.15).

PC	Variable 1	Variable 2	*Paired samples t-tests*					
			R. hipposideros			***Both species***		
			t	**df**	**P**	***t***	**df**	**P**
1	*Pre-treatment*	*Post-treatment 1 and 2*	-0.693	3	>0.05	-1.395	*5*	*>0.05*
	Post-treatment 1 and 2	*Post-treatment 3*	0.238	2	>0.05	-0.039	*3*	*>0.05*
	Pre-treatment	*Post-treatment 3*	-0.088	2	>0.05	-0.710	*3*	*>0.05*
2	*Pre-treatment*	*Post-treatment 1 and 2*	-0.083	3	>0.05	0.074	*5*	*>0.05*
	Post-treatment 1 and 2	*Post-treatment 3*	0.637	2	>0.05	0.666	*3*	*>0.05*
	Pre-treatment	*Post-treatment 3*	0.209	2	>0.05	0.170	*3*	*>0.05*
3	*Pre-treatment*	*Post-treatment 1 and 2*	-0.620	3	>0.05	-1.486	*5*	*>0.05*
	Post-treatment 1 and 2	*Post-treatment 3*	0.401	2	>0.05	-0.476	*3*	*>0.05*
	Pre-treatment	*Post-treatment 3*	-0.590	2	>0.05	-1.302	*3*	*>0.05*
4	*Pre-treatment*	*Post-treatment 1 and 2*	2.382	3	>0.05	1.208	*5*	*>0.05*
	Post-treatment 1 and 2	*Post-treatment 3*	0.527	2	>0.05	0.445	*3*	*>0.05*
	Pre-treatment	*Post-treatment 3*	1.175	2	>0.05	0.887	*3*	*>0.05*

Table 4.15 The results of paired samples t-tests between each treatment category for *R. hipposideros* and for *R. hipposideros* and *P. auritus* combined.

4.12.2 Trends across three treatment categories (Post-treatment samples 1 & 2 combined)

Principle component 1

Average principle component 1 (PC 1) scores increased after exposure to artificial lighting in the previous 7-14 days but decreased slightly following seven days without artificial lighting (Figure 4.14 a). These patterns indicate that corticosteroids initially decreased whilst reproductive hormones increased, but that following a week without lighting corticosteroids increased whilst reproductive hormones decreased.

PC 1 scores amongst *P. auritus* increased in each subsequent treatment category (i.e. corticosteroids continued to decrease whilst reproductive hormones increased; Figure 4.15 a); there was only one sample for post-treatment 3 which meant that an average and the standard error could not be calculated) whereas *R. hipposideros* average scores increased after exposure to artificial lighting but decreased following seven days without artificial lighting (Figure 4.16 a).

Principle component 2

Average scores for principle component 2 (PC 2) decreased in each subsequent treatment category (Figure 4.14 b). This trend was also observed for *P. auritus* (Figure 4.15 b) and *R. hipposideros* (Figure 4.16 b). These results indicate that two corticosteroids (cortisone and hydrocortisone) decreased whilst one (11-deoxycorticosterone) increased.

Principle component 3

Average scores for principle component 3 (PC 3) increased in each subsequent category (Figure 4.14 c). *P. auritus* followed this trend (Figure 4.15 c). Thus, two reproductive hormones (estrone and 17β-estradiol) increased over time.

R. hipposideros scores increased after exposure to artificial lighting in the previous 7-14 days but decreased following seven days without artificial lighting (Figure 4.16 c), consequently, two reproductive hormones increased and then decreased.

Principle component 4

The average score for principle component 4 (PC 4) decreased in each subsequent category (Figure 4.14 d). *P. auritus* scores increased in each subsequent category (Figure 4.15 d) whereas *R. hipposideros* average scores decreased (Figure 4.16 d).

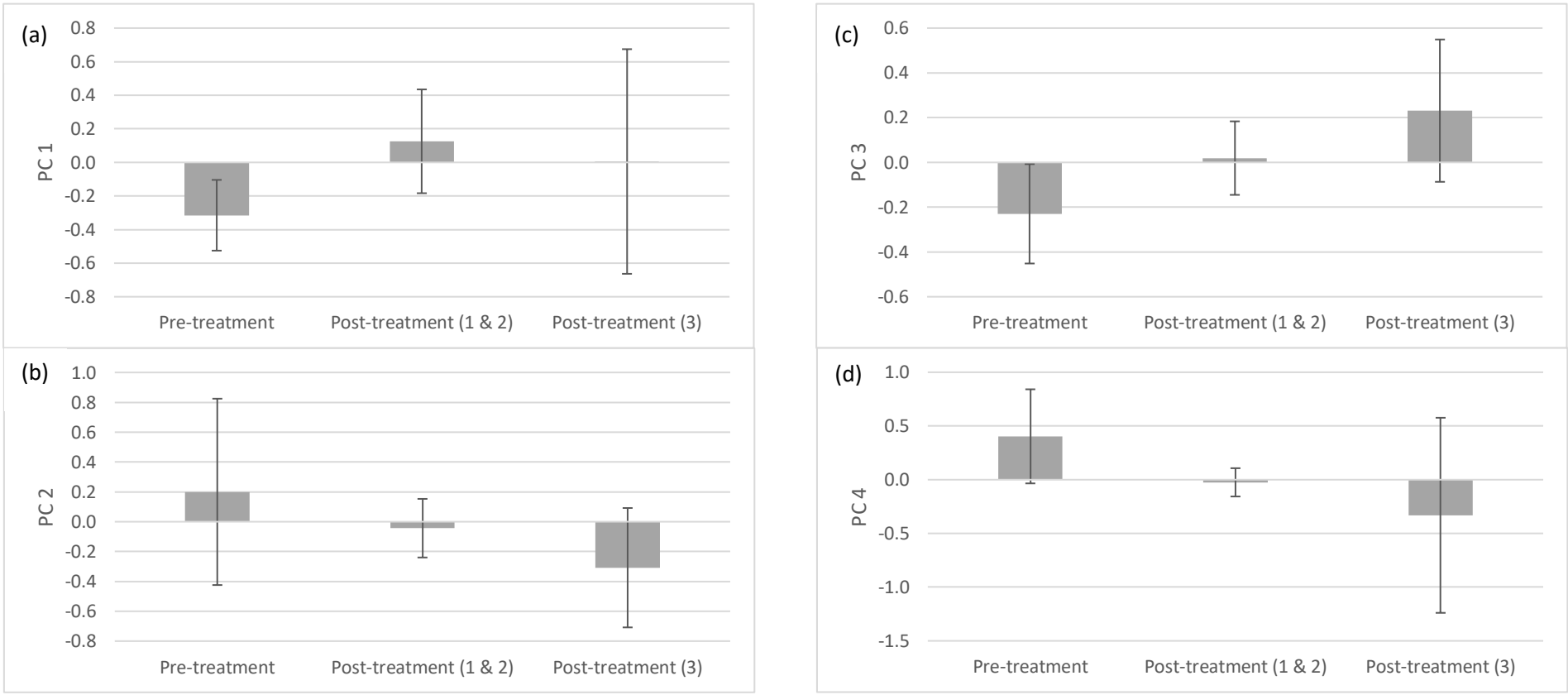


Figure 4.14 Average principle component scores for *R. hipposideros* and *P. auritus* combined, for PC 1 (a), PC 2 (b), PC 3 (c), PC 4 (d). Post-treatment 1 & 2 samples have been combined.

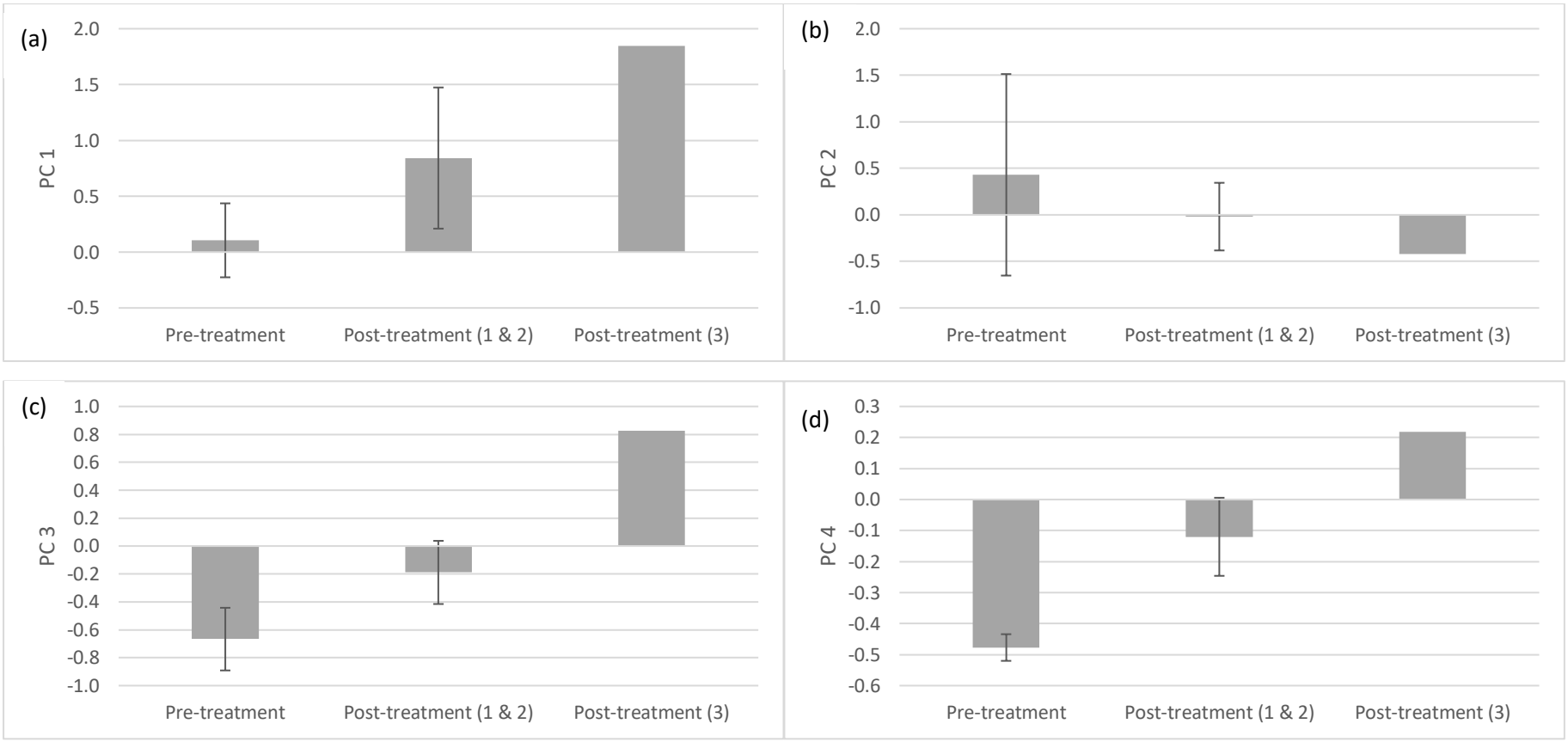


Figure 4.15 Average principle component scores for *P. auritus*, for PC 1 (a), PC 2 (b), PC 3 (c), PC 4 (d). Post-treatment 1 & 2 samples have been combined. There was only one sample for post-treatment score 3 which meant that an average and standard error could not be calculated.

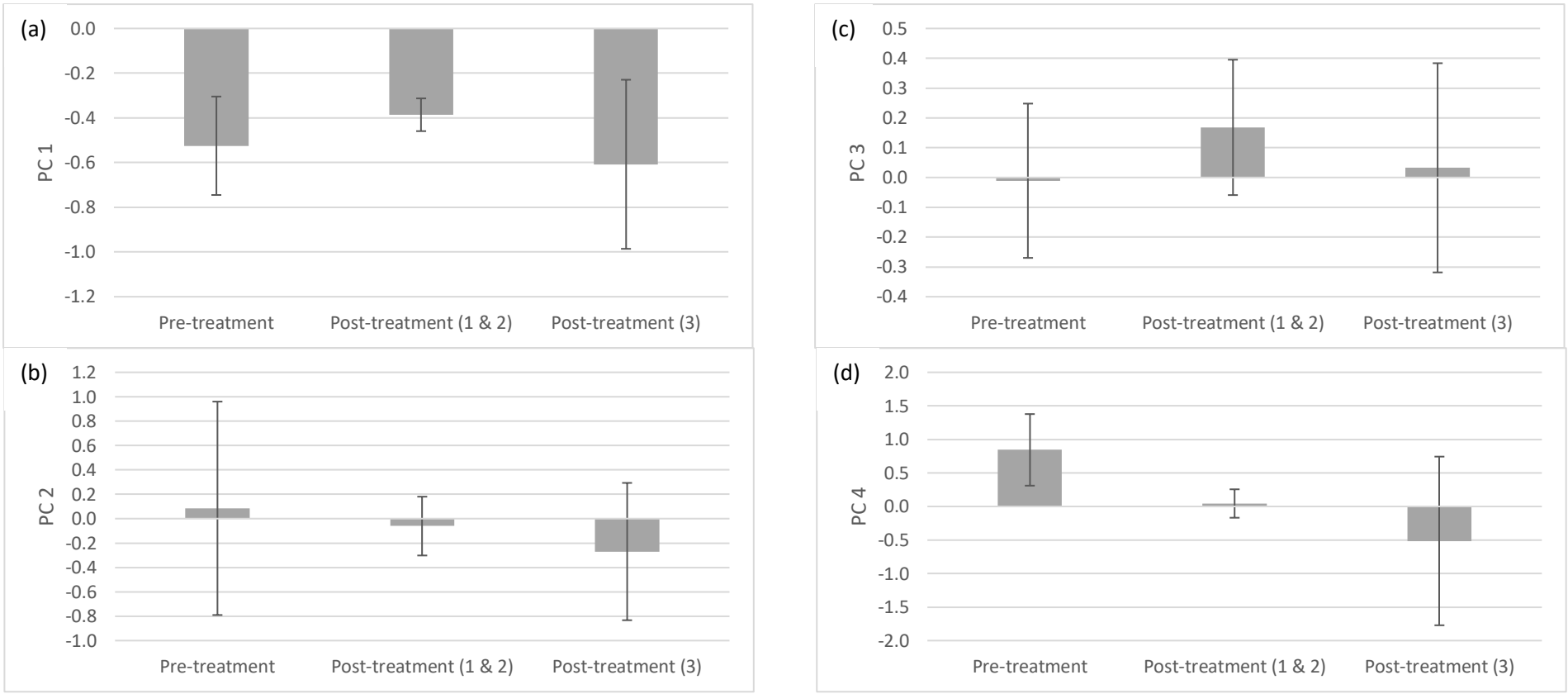


Figure 4.16 Average principle component scores for *R. hipposideros*, for PC 1 (a), PC 2 (b), PC 3 (c), PC 4 (d). Post-treatment 1 & 2 samples have been combined.

4.12.3 Differentiation of species and treatments by ordination

Principle component 1

P. auritus and *R. hipposideros* differentiate for PC 1; *P. auritus* scores tend to be higher than *R. hipposideros* with some overlap at central scores (Figure 4.17 a). *P. auritus* post-treatment samples tend to be higher than their pre-treatment samples whereas *R. hipposideros* pre-treatment samples fall within the range of their post-treatment samples.

Principle component 2

Overall, there is a great deal of overlap between *P. auritus* and *R. hipposideros* scores for PC 2 (Figure 4.17 a). Within species, pre-treatment and post-treatment scores are also within the same range.

Principle component 3

R. hipposideros and *P. auritus* scores are largely overlapping for PC 3 (Figure 4.17 b). Within species, pre-treatment and post-treatment scores are within the same range.

Principle component 4

P. auritus and *R. hipposideros'* scores overlap for PC 3. *R. hipposideros* pre-treatment and post-treatment scores overlap (Figure 4.17 b), but pre-treatment scores remain above 0 whereas several post-treatment scores are below 0. *P. auritus* pre-treatment scores are slightly lower than post-treatment scores. Overall, I found that hormone profiles changed over time, but generally appeared that they were unresponsive to experimental treatment lighting.

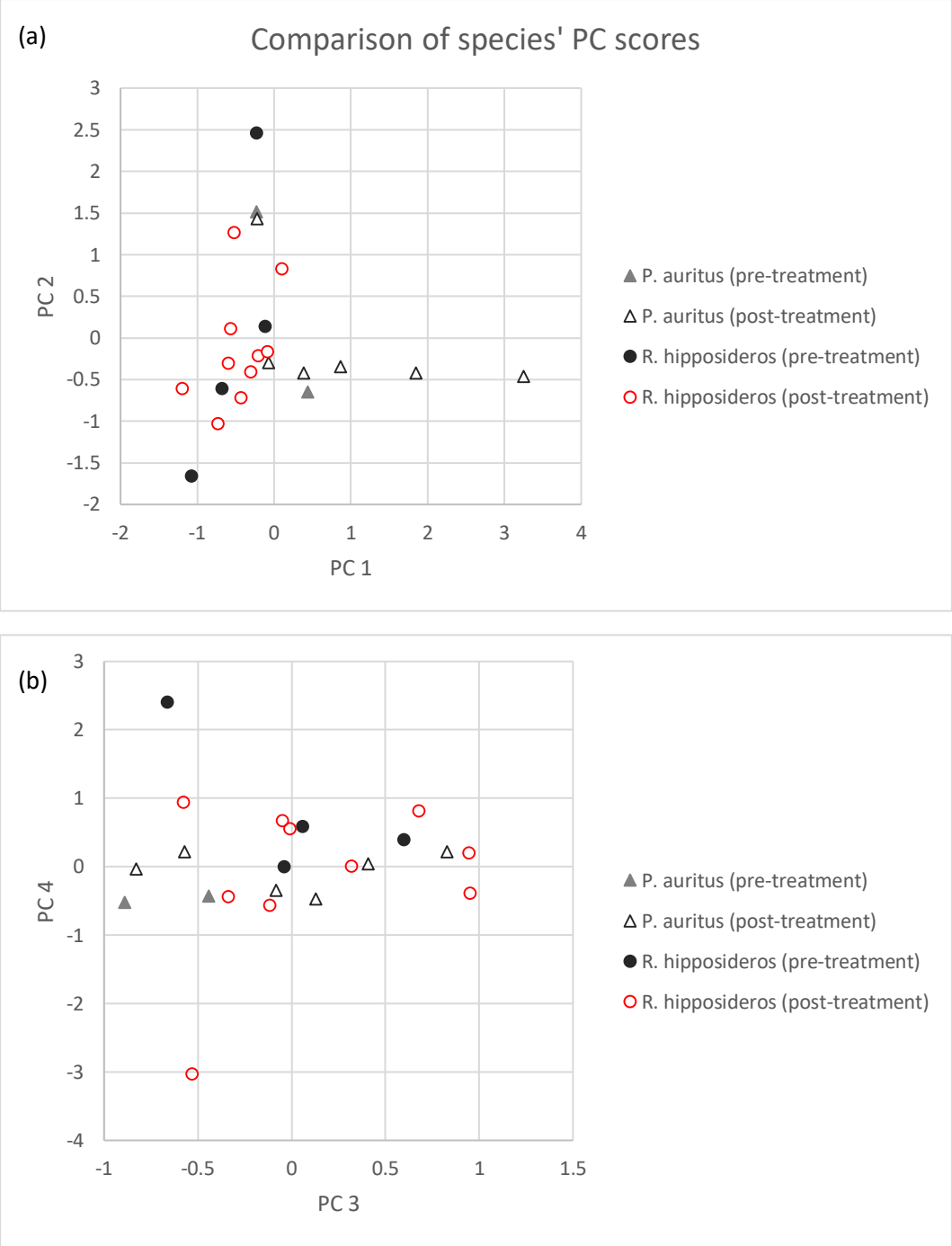


Figure 4.17 *P. auritus* and *R. hipposideros* scores for PC 1 plotted against PC 2 (a) and PC 3 against PC 4 (b).

4.13 Discussion of experimental study

There were no significant differences between treatment categories for any of the principle components for *R. hipposideros* or *P. auritus* or both species combined. However, there are trends across treatment categories. When considering both species combined, PC 1 and PC 3 increased between treatment categories, whilst PC 2 and PC 4 decreased between categories. These trends across principle components indicate that glucocorticoids generally decreased whilst reproductive hormones increased over time.

In hibernating species, glucocorticoids increase towards the end of the summer and into autumn in order to facilitate hyperphagia, enabling the organism to accumulate fat stores in preparation for winter (Reeder et al. 2004). That glucocorticoids decline at a time when we might expect an increase suggests the downward trend in glucocorticoids following exposure to artificial lighting could be as a result of downregulation of the HPA axis due to chronic stress.

Given that treatments were assigned sequentially, an alternative and possibly more convincing explanation is that the results presented here appear to simply reflect seasonal changes in corticosteroids and reproductive hormones. *R. hipposideros* and *P. auritus* breed in autumn, shortly after the end of the sampling period.

Had I included control roosts in my experimental design and had sampled both treated and control roosts for a longer period of time (perhaps until maternity roosts had disbanded) it might have been possible to distinguish between these two hypothesise.

4.14 Individuals, sexes and species

4.14.1 Introduction

There are many sources of variation which may be expressed as differences in glucocorticoid concentrations in samples taken at different stages within an individual's life, between individuals within a population and between sexes, populations and species (Cockrem 2013; Goyman 2012; Romero 2002; Romero 2004).

These sources of variation may be summarised as being due to inherent differences in physiology and variation in the capacity to modify the HPA axis during the course of an organism's life (section 4.3.3; Cyr & Romero 2009); differences in energetic demands placed on the organism during diurnal, seasonal and life history stages, determined in part by the ecology of the species (section 4.3.2; Romero 2002); and differences in the availability of energy in the environment to support energetic demands (section 4.4; McEwen & Wingfield 2003; Ellis et al. 2012).

Here I will focus on some of the sources of variation that could explain the substantial variation in glucocorticoid levels often observed amongst individuals, and differences between sexes and species.

4.14.2 Species

Basal, modulated and stress related levels of glucocorticoids are likely to vary between species as a result of differences in the physiology of the HPA axis. For example, there may be differences in the number and placement of glucocorticoid and mineralocorticoid receptors. The number and placement of receptors influences tissue

sensitivity to circulating levels of glucocorticoids, which in turn determines appropriate basal and modulated levels as well as the “tipping point” where the ELHS is triggered.

Glucocorticoid responses are under genetic control; lines of rainbow trout and Japanese quail have been selected for low and high glucocorticoid responses (Cockrem 2013). This suggests that populations living in very different environments and exposed to different types of stressor, for example populations expanding into urban areas, could potentially evolve (i.e. the frequency of alleles relating to the phenotypic expression of the stress response within a population changes over time) so that the HPA axis operates at appropriate baseline and stress induced levels. Genetic and consequently physiological differences may account for the extremely different baseline glucocorticoid levels observed in different taxonomic groups (Cockrem 2013; Romero 2004).

However, even closely related species may have different modulated baseline levels of glucocorticoids owing to differences in their ecology; e.g. differences in preferred food type, reproductive timing, whether they hibernate or not.

4.14.3 Individuals

The number and placement of receptors is plastic, i.e. an individual’s experiences could result in a downregulation of the GR or MR receptors. Therefore, stress related elevations in glucocorticoids are likely to differ amongst individuals owing to behavioural and experiential (pre-, post-natal and adult; Cockrem 2013) differences that influence the strength of the stress response and mediate acclimation, habituation or sensitization.

Receptor plasticity, mediated by pre-, post-natal and adult experiences, may be a component of personality. A great deal of variation in the stress response between individuals was revealed in a review of capture and restraint studies. Cockrem et al. (2013) calculated the coefficient of variation for glucocorticoid responses at different time scales after exposure to a stressor (capture and restraint). In mammals, CV remained high at 58.5% at the initial sample and 58% at 1-4 hours. This variation is suggestive of individual responses to stress being a component of personality; a behavioural syndrome consistent within an individual and varied within a population. Animals with proactive or bold personalities tend to have lower glucocorticoid responses than animals with shy or reactive personalities.

Glucocorticoids levels also vary with age. In bats, FGMs increase with age, consistent with other studies. Neonates have a supressed HPA axis (hyporesponsive; Reeder and Kramer 2005) and the strength of the negative feedback system mediated by glucocorticoids appears to grow weaker with age, i.e. older individuals take longer to recover from stressors.

Differences in diet can also have an effect on corticosteroid concentration yields. The more fibre there is in an animal's diet, the longer it takes for a meal to pass through the digestive tract. This allows more faecal corticosteroids and metabolites to be reabsorbed from the gut and more time for microbial action to break down compounds.

4.14.4 Sex differences

Males and females often have different baseline and stress related glucocorticoid levels. In mammals, females usually have higher baseline

glucocorticoids (Reeder and Kramer 2005). The female stress response is more variable; sometimes it is more robust than males and other times less so, possibly reflecting other sex-specific seasonal cycles such as territoriality and mate guarding (males) or pregnancy and lactation (females). In bats, males and females often have similar glucocorticoid levels outside of breeding season, though there are exceptions (Table 4.3).

Males may have seasonal peaks in glucocorticoids during the breeding season, possibly to support the energetic demands of finding a mate, mate guarding, territorial behaviour and other forms of male-male competition (Balm 1999, Reeder & Kramer 2005). On the other hand, in species where female mate choice or male-male competition requires optimal body condition, it is possible that the HPA axis is downregulated in order to avoid the costs associated with long-term exposure to glucocorticoids such as a reduction in body mass (Fletcher et al. 2015).

In a variety of female mammals, glucocorticoids increase during the middle and late gestation period and decline at parturition. In little brown myotis, baseline glucocorticoids were elevated in females during mid to late pregnancy relative to early pregnancy and lactation and relative to glucocorticoid levels in males (Reeder and Kramer 2005) and in Brazilian free-tailed bats, cortisol levels were significantly higher during pregnancy and lactation (Allen et al. 2010). Whilst baseline levels increase during late pregnancy, stress induced levels can be decreased during pregnancy and lactation. Downregulation of the stress response could also be beneficial during pregnancy owing to the potentially negative impacts of glucocorticoids on the foetus, and the body condition of pregnant females (Reeder and Kramer 2005).

The purpose of this study was to explore variation amongst individuals and to compare species and sexes within species in order to confirm that the methodology for extraction of target compounds can identify species and sex differences and to validate decisions made with regards to the correlative and experimental study.

4.15 Methods

4.15.1 Collection of faecal samples

Fieldwork took place at Wytham Woods, Oxfordshire, between May and early October of 2013 and 2014. There are 1265 boxes located throughout Wytham Woods (August et al. 2014). Bats from several species roost in these boxes during the day. Faecal samples were taken directly from individuals of two species; *M. daubentonii* and *M. nattereri*. Bats were held in the hand and as they defecated, the faecal pellet was collected in an Eppendorf. Each Eppendorf tube was stored with ice-packs for up to 6 hours before being frozen at -20°C. The species, sex and approximate age (e.g. juvenile, adult) of each individual was noted. In total, faecal samples were collected from 57 *M. daubentonii* individuals, of which 31 were female and 26 were male; and 57 *M. nattereri* individuals of which 37 were female and 20 were male. All samples came from adult individuals.

4.15.2 Extraction of target compounds and sample preparation for LC-MS QQQ analysis

Individual faecal samples were freeze-dried to remove water before being ground and homogenized (Bead Mill Homogenizer; BioSpec, USA). 600µl of methanol

were added to the Eppendorf's containing the dried faecal sample. Samples were homogenized with a vortex (Vortex Mixer; Alpha Laboratories, UK) and sonicated to further break down faecal matter. Each sample was then centrifuged at 13,000 rpm for 10 minutes. 500 µl of supernatant was transferred to a new Eppendorf with 500µl of 1% formic acid. Each sample was then pipetted on to an SLE+ isolute column (1ml; Biotage, Sweden) attached to a vacuum pump and washed through with 2.5ml ethyl acetate twice as per the manufacturer's instructions. The filtered sample was collected in a falcon tube and dried under nitrogen gas before being stored at -80C.

Samples were re-suspended in 500µl of 50% methanol, 50% water, 0.1% formic acid spiked with umbelliferone. The umbelliferone served as an internal standard. The sample was then vortexed for 20 seconds and transferred into new Eppendorf tubes which were centrifuged for 5 minutes. The whole sample was then filtered into MS glass vials through a 0.45 µl filter tip.

4.15.3 Quantitative LC-MS QQQ analysis and mass spectrum data analysis

Quantitative analysis of hormones was performed using an Agilent 6420B triple quadrupole (QQQ) mass spectrometer (Technologies, Palo Alto, USA) hyphenated to a 1200 series Rapid Resolution HPLC system. 10 µl of sample extract were loaded onto an Eclipse Plus C18 3.5 µm, 2.1 x 150 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA). For detection using positive ion mode, mobile phase A comprised of 100% LC-MS grade H2O, with 0.1% Formic Acid and mobile phase B was 100% Methanol (LC-MS grade) with 0.1% Formic Acid. The following gradient was used: 0 min – 40% B; 20 min – 100% B; 25 min – 100% B; 26 min – 40% B followed by 4 min re-equilibration time. The flow rate was 0.25 mL min^{-1} and the

column temperature was held at 35 °C for the duration. The QQQ source conditions for electrospray ionisation were as follows: gas temperature 350 °C, drying gas flow rate of 11 l min-1, nebuliser pressure 35 psig, and capillary voltage 4 kV. All ions were scanned in positive ion mode and given a dwell time of 30 mseconds. The fragmentor voltage and collision energies had previously been optimised for each compound (Appendix 4.3). A 10uM mix containing each of these hormone standards was diluted by half each time to produce a concentration range from 10uM to 0.156uM. A blank (extraction solvent spiked with umbelliferone) was run every 6 samples, and a 0.156 uM hormone mix every 12 samples as a quality control.

Data analysis was undertaken using Agilent Mass Hunter Quantitative analysis software for QQQ (Version B.07.01). Concentrations were calculated using calibration curves generated for each of the hormone standards (Appendix 4.42). Concentrations were standardised by sample weight.

4.15.4 Statistical analysis

I compared the median concentration of each target compound for each species and between males and females within each species with a Mann-whitney U test. Due to high levels of intercorrelation between target compounds, I conducted variable reduction (principle components analysis). Intercorrelations between target compounds were tested with Pearson's correlation coefficient and Spearman's rank correlation. Principle component (PC) scores for each species and for both sexes within species were plotted against each other (e.g. PC 1 against PC 2 and PC 3 against PC 4) in order to determine whether species and sexes differentiated from each other in terms of their component scores. I then compared PC scores between

3007 species and between males and females within each species with the Mann-Whitney
3008 U test.

3009 4.16 Results

3010 Twelve out of 15 target compounds were identified in faecal samples (Table 4.16).

Target compound	**Primary role**
Aldosterone	Corticosteroid
Corticosterone	Corticosteroid
Cortisone	Corticosteroid
Hydrocortisone	Corticosteroid
11-deoxycorticosterone	Corticosteroid
Androsterone	Reproductive hormone
Dihydrotestosterone	Reproductive hormone
Progesterone	Reproductive hormone
Testosterone	Reproductive hormone
1-dehydrotestosterone	Reproductive hormone
Pregnenalone	Precursor to both reproductive hormones and corticosteroids
17α-hydroxyprogesterone	Precursor to both reproductive hormones and corticosteroids

Table 4.16 A list of target compounds including corticosteroids and reproductive hormones or their metabolites and precursors to both reproductive hormones and corticosteroids.

4.16.1 Principle component analysis

Target compounds were highly intercorrelated (Table 4.17). The strongest positive correlations were between compounds positioned closely on the steroid hormone biosynthesis pathway (see Appendix 4.6). Principle component analysis produced four distinct components (Table 4.18) with Eigen values above 1 (Figure 4.16; Table 4.19).

	Aldosterone		Androsterone		Corticosterone		Cortisone		Dihydrotestosterone		Hydrocortisone		Pregnenalone		Progesterone		Testosterone		1-dehydrotestosterone		11-deoxycorticosterone	
Androsterone	.368**	.419**																				
Corticosterone	.311**	.710**	.323**	.326**																		
Cortisone	.885**	.387**	.318**	.229*	.145	.385**																
Dihydrotestosterone	.094	.441**	.157	.393**	.345**	.508**	.092	.293**														
Hydrocortisone	.143	.353**	.344**	.195*	.677**	.398**	.067	.440**	.170	.338**												
Pregnenalone	.225*	.498**	.028	.124	.504**	.468**	.084	.340**	.295**	.288**	.277**	.198*										
Progesterone	.187*	.485**	.140	.204*	.226*	.401**	.022	.384**	.096	.281**	-.015	.267**	.116	.384**								
Testosterone	.893**	.233*	.439**	.588**	.157	.279**	.943**	.177	.073	.301**	.070	.161	.016	.010	.049	.159						
1-dehydrotestosterone	.648**	.566**	.578**	.541**	.469**	.522**	.593**	.361**	.135	.298**	.412**	.337**	.109	.252**	.120	.345**	.665**	.677**				
11-deoxycorticosterone	.426**	.551**	.208*	.313**	.601**	.493**	.227*	.235*	.080	.303**	.509**	.289**	.426**	.343**	.139	.471**	.202*	.191*	.381**	.428**		
17α-hydroxyprogesterone	.903**	.504**	.333**	.256**	.117	.461**	.961**	.340**	.031	.222*	.026	.433**	.027	.307**	.022	.443**	.972**	.194*	.591**	.434**	.194*	.317**

Table 4.17 The results of intercorrelations between target compounds. For each intercorrelation, the result of Pearson's correlation is reported on the left side of the column and the result of Spearman's correlation is reported on the right. Results highlighted in light pink squares are significant (<0.05) and those highlighted in dark pink are highly significant (<0.01).

Target compound	Role	PC 1	PC 2	PC 3	PC 4
		(41.16 %)	(20.34 %)	(9.12 %)	(8.46 %)
Aldosterone	Stress	.921			
Corticosterone			.811	.314	
Cortisone		.967			
Hydrocortisone (cortisol)			.721	.514	
11-deoxycorticosterone			.765		
Androsterone	Sex			.796	
Dihydrotestosterone			.316		.521
Progesterone					.857
Testosterone		.961			
1-dehydrotestosterone		.579		.588	
Pregnenalone	Precursor to both stress and sex hormones		.746	-.347	
17α-hydroxyprogesterone		.981			

Table 4.18 Rotated component loadings for each principle component. The percentage variance that each component accounts for is provided in brackets.

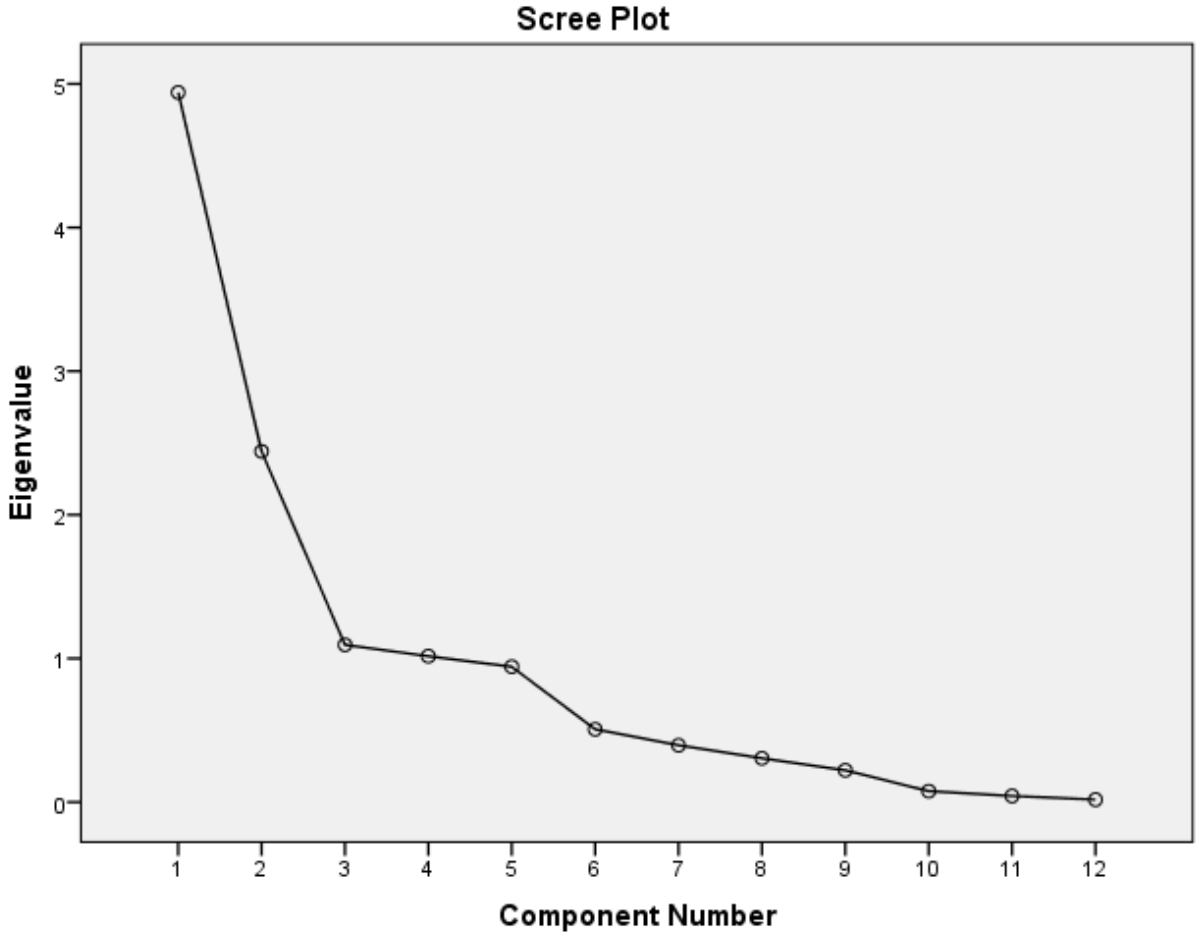


Figure 4.18 Scree plot of Eigen values for principle components.

Component	Eigen value	% of variance	Cumulative variance
1	4.940	41.169	41.169
2	2.442	20.348	61.517
3	1.095	9.125	70.641
4	1.016	8.465	79.106

Table 4.19 The Eigen value of each principle component as well as the proportion of variance accounted for by each principle component and cumulative variance.

Principle component 1 (PC 1) accounts for 41% of variance (Table 4.19) and is composed of two stress related compounds (aldosterone and cortisone) and two sex related compounds (testosterone and 1-dehydrotestosterone) as well as one precursor to both stress and sex related compounds (17α-hydroxyprogesterone; Table 4.18). All compounds are moderately (0.5) or strongly (0.9) positively correlated with PC 1, so that as PC 1 increases, all compounds increase.

Principle component 2 (PC 2) accounts for 20% of variance (Table 4.19) and is composed of three stress related compounds (corticosterone, cortisone and hydrocortisone), one sex related compound (dihydrotestosterone) and one precursor to both stress and sex related compounds (pregnenalone; Table 4.18). All compounds were positively correlated with PC 2, so that as PC 2 increases, all compounds increase. However, stress related compounds were more strongly correlated with PC 2 (all above 0.7) than the single sex related compound (dihydrotestosterone, 0.3).

Principle component 3 (PC 3) accounted for 9% of variance (Table 4.19) and was composed of two stress related compounds (corticosterone and hydrocortisone), two sex related compounds (androsterone and 1-dehydrotestosterone) and one precursor to both stress and sex related compounds (pregnenalone; Table 4.18). All stress and sex related compounds are positively correlated with PC 3, so that as PC 3 increases these compounds increase. The precursor to both stress and sex related compounds (pregnenalone) is negatively correlated with PC 3 and decreases as PC 3 increases.

Principle component 4 (PC 4) accounted for 8% of variance (Table 4.19) and was composed of two sex related compounds (dihydrotestosterone and progesterone). Both compounds were either moderately (dihydrotestosterone, 0.5) or

highly (progesterone, 0.8) positively correlated with PC 4, so that as PC 4 increases, both compounds increase (Table 4.18).

Ordination plots reveal that there is a great deal of overlap between species and sexes for principle components 1, 2 and 4 (Figure 4.19 a and b). There are outliers for PC 1 (a male *M. daubentonii*), PC 3 (a male *M. nattereri)* and PC 4 (a female *M. daubentonii*). There is more differentiation between species for PC 3, especially between female *M. daubentonii* and female *M. nattereri*. Generally, there is more variation amongst females in both species.

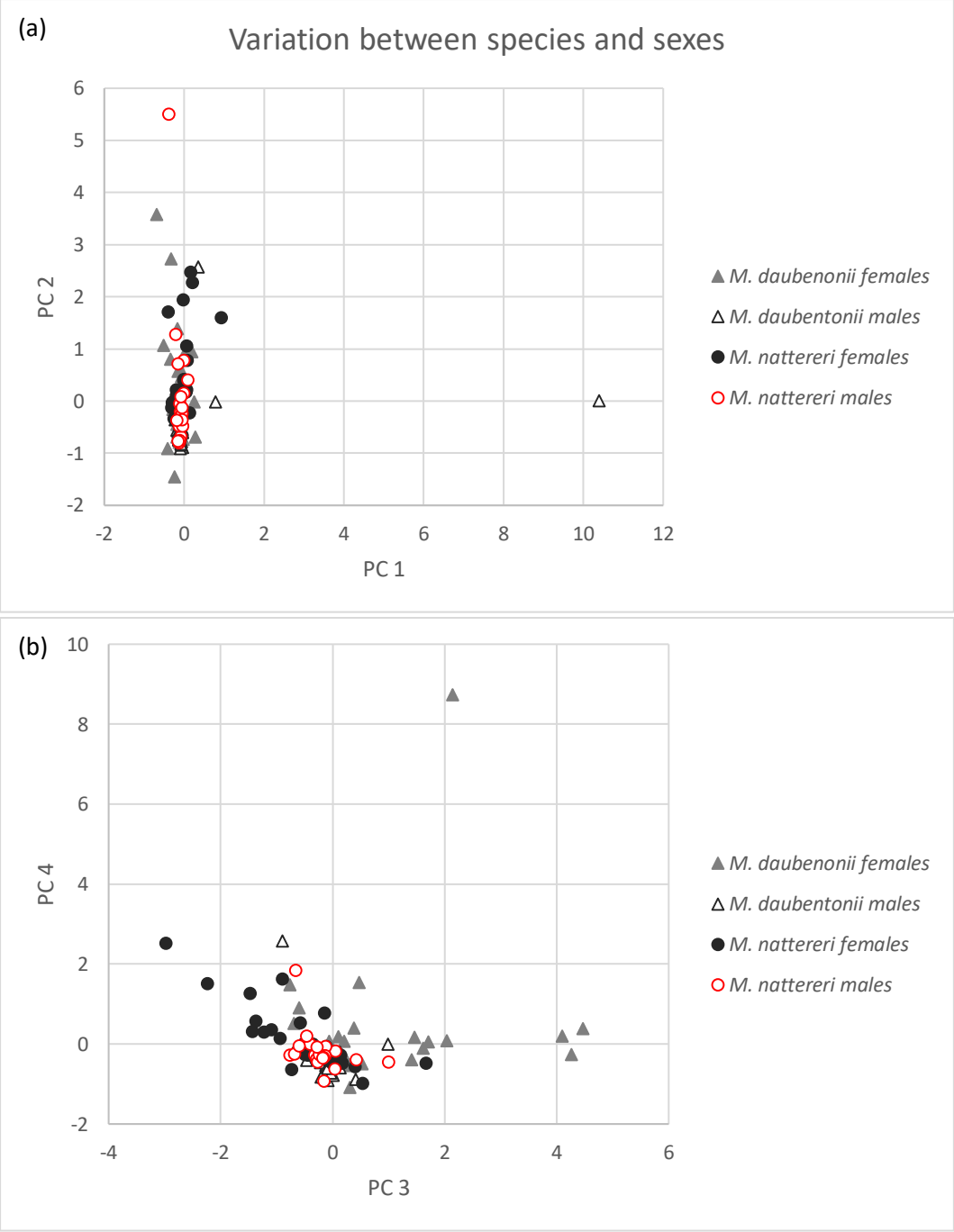


Figure 4.19 Male and female *M. daubentonii* and *M. nattereri* scores for PC 1 plotted against PC 2 (a) and PC 3 against PC 4 (b).

4.16.2 Comparisons between species

Target compounds

M. daubentonii had higher levels of three reproductive hormones (androsterone, testosterone and 1-dehydrotestosterone) than *M. nattereri* whereas *M. nattereri* had higher levels of a precursor to both reproductive hormones and corticosteroids (pregnenalone) than *M. daubentonii* (Table 4.20; Appendix 4.45).

Principle components

M. nattereri had a higher average score for PC 2 than *M. daubentonii* (Figure 4.19 a) whereas *M. nattereri* had a lower average score for PC 3 than *M. daubentonii* (Figure 4.20 b; Table 4.21). There was no significant difference in the average scores of *M. daubentonii* and *M. nattereri* for PC 1 and PC 4 (Table 4.21; Appendix 4.46).

These results are reflected in the ordination plots. Most individuals from both species fall within a narrow range for PC 1 scores, with one extreme outlier from *M. daubentonii* (Figure 4.21 a). Individuals from both species score a wide range of mostly overlapping scores for PC 2 with one moderate outlier from *M. nattereri* (Figure 4.21 a). There is clearly more differentiation between species for PC 3; *M. daubentonii* individuals tend to have higher scores than *M. nattereri* with some overlap at central scores (Figure 4.21 b). *M. daubentonii* and *M. nattereri* overlap within a narrow range of scores for PC 4, with one outlier from *M. daubentonii* (Figure 4.21 b).

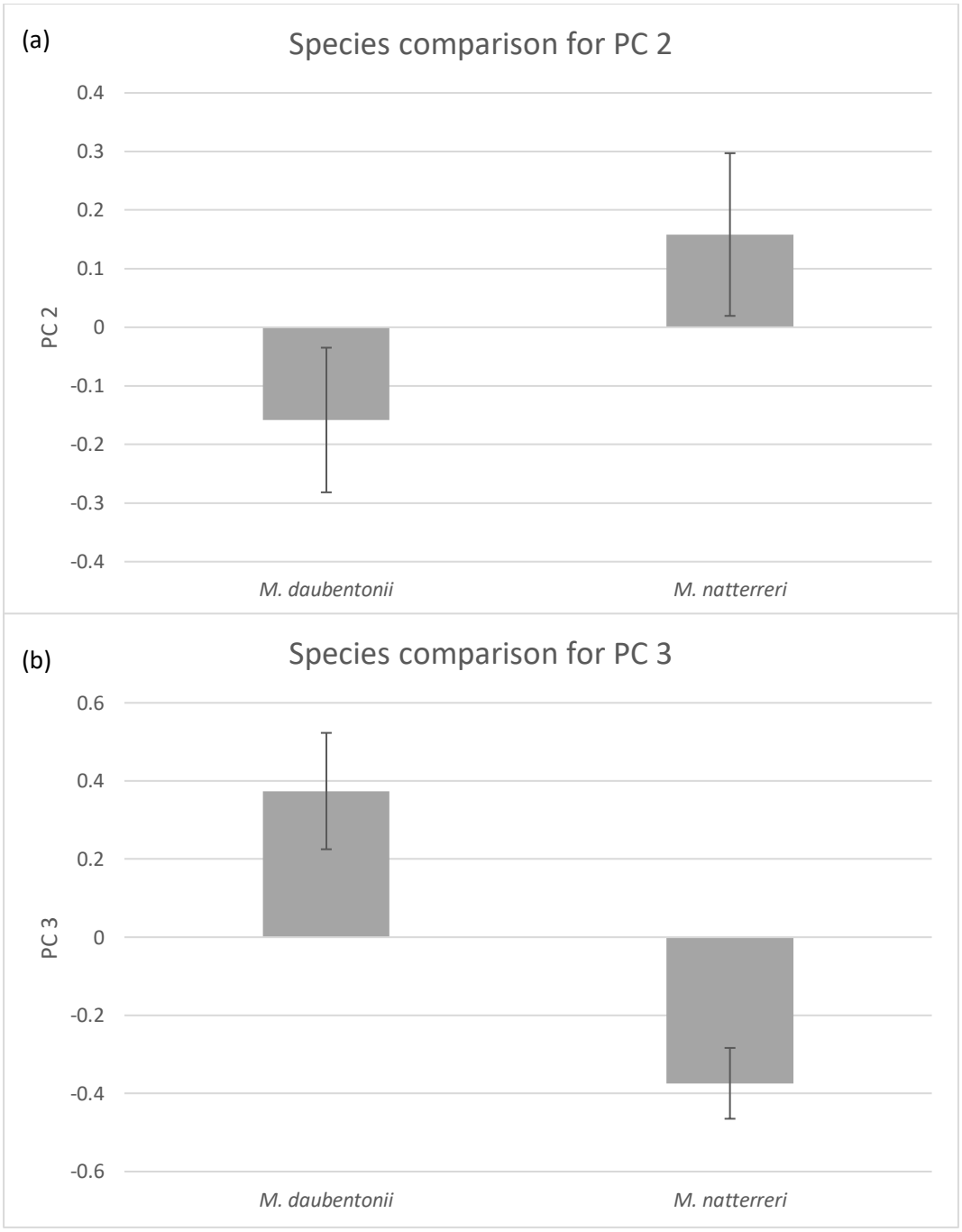


Figure 4.20 A comparison of *M. daubentonii* and *M. nattereri* average scores for PC2 (a) and PC 3 (b).

Comparison of species	Mean				Median			
	Md	***SEM***	***Mn***	***SEM***	***Md***	***Mn***	***U***	**p-value**
Aldosterone	0.021	0.0073	0.014	0.0028	0.008	0.009	1565.000	.736
Androsterone	0.012	0.003	0.002	0.0003	0.003	0.001	1021.000	.001***
Corticosterone	0.001	0.0001	0.001	0.0001	0.001	0.001	1557.000	.702
Cortisone	0.001	0.0002	0.001	0.0001	0.001	0.001	1399.000	.201
Dihydrotestosterone	0.007	0.001	0.006	0.0011	0.004	0.003	1513.000	.527
Hydrocortisone	0.001	0.0001	0.001	0.0001	0.001	0.001	1470.000	.381
Pregnenalone	0.012	0.0018	0.021	0.0028	0.008	0.015	1026.000	.001***
Progesterone	0.001	0.0004	0.001	0.0002	0.001	0.001	1590.000	.845
Testosterone	0.003	0.0011	0.001	0.0001	0.001	0.001	539.000	<0.0001****
1-dehydrotestosterone	0.001	0.0001	0.001	0.0001	0.001	0.001	856.000	<0.0001****
11-deoxycorticosterone	0.002	0.0004	0.003	0.0007	0.001	0.001	1369.000	.148
17α-hydroxyprogesterone	0.009	0.0082	0.001	0.0002	0.001	0.001	1615.000	.957

Table 4.20 The results of Mann-Whitney U tests on comparisons between *M. daubentonii* (Md) and *M. nattereri* (Mn) for each target compound (µM/mg).

	Mean ranks		Sum of ranks		Mann-Whitney U test	
Comparison of species						
PC	***M. daubentonii*** **(n=57)**	***M. nattereri*** **(n=57)**	***M. daubentonii*** **(n=57)**	***M. nattereri*** **(n=57)**	***U***	**p-value**
1	56.70	58.30	3232	3323	1579.000	0.797
2	48.39	66.61	2758	3797	1105.000	<0.01**
3	72.58	42.42	4137	2418	765.000	<0.0001****
4	57.42	57.58	3273	3282	1620.000	0.980
Comparison of sexes						
M. daubentonii						
PC	**Female** **(n=31)**	**Male** **(n=26)**	**Female** **(n=31)**	**Male** **(n=26)**	***U***	**p-value**
1	25.94	32.65	804	849	308.000	0.128
2	32.48	24.85	1007	646	295.000	0.084
3	35.42	21.35	1098	555	204.000	0.001***
4	31.65	25.85	981	672	321.000	0.189
M. nattereri						
PC	**Female** **(n=37)**	**Male** **(n=20)**	**Female** **(n=37)**	**Male** **(n=20)**	***U***	**p-value**
1	28.76	29.45	1064	589	361.000	0.880
2	30.35	26.50	1123	530	320.000	0.403
3	27.65	31.50	1023	630	320.000	0.403
4	30.46	26.30	1127	526	316.000	0.367

Table 4.21 The results of Mann-Whitney U tests on comparisons between *M. daubentonii* and *M. nattereri* and between males and females within each species for each principle component.

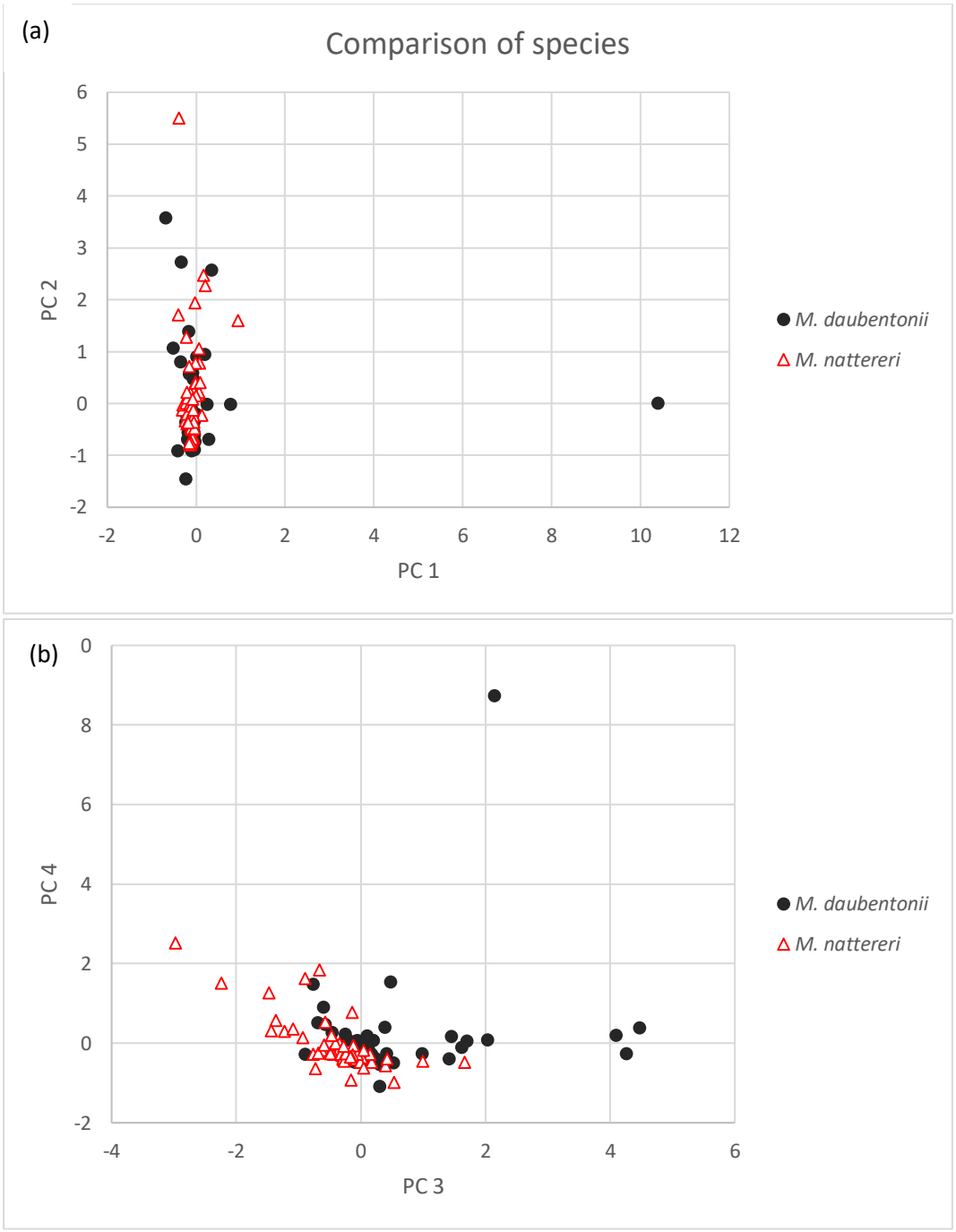


Figure 4.21 *M. daubentonii* and *M. nattereri* scores for PC 1 plotted against PC 2 (a) and PC 3 against PC 4 (b).

4.16.3 Comparisons between male and female *M. daubentonii*

Target compounds

M. daubentonii females had higher levels of one corticosteroid (hydrocortisone) and three reproductive hormones (dihydrotestosterone, 1-dehydrotestosterone, progesterone; Table 4.22) than males whilst *M. daubentonii* males had higher levels of two corticosteroids (aldosterone and corticosterone) and two reproductive hormones (androsterone and dihydrotestosterone; Table 4.22) than females (Appendix 4.45).

Principle components

M. daubentonii females had a higher average score for PC 3 than males (Figure 4.22). There were no significant differences between male and female average scores for PC 1, PC 2 or PC 4 (Table 4.21; Appendix 4.46). Ordination plots show that most males and females fall within a narrow range of scores for PC 1 but with one male outlier (Figure 4.23 a). Males and females cluster between 0 and -1 for PC 2 although there is more variation in females (Figure 4.23 a). Similarly, males and females cluster at lower scores for PC 3 but again there is more variation amongst females (Figure 4.23 b). Males and females fall within a narrow range of scores for PC 4 but with one female outlier (Figure 4.23 b).

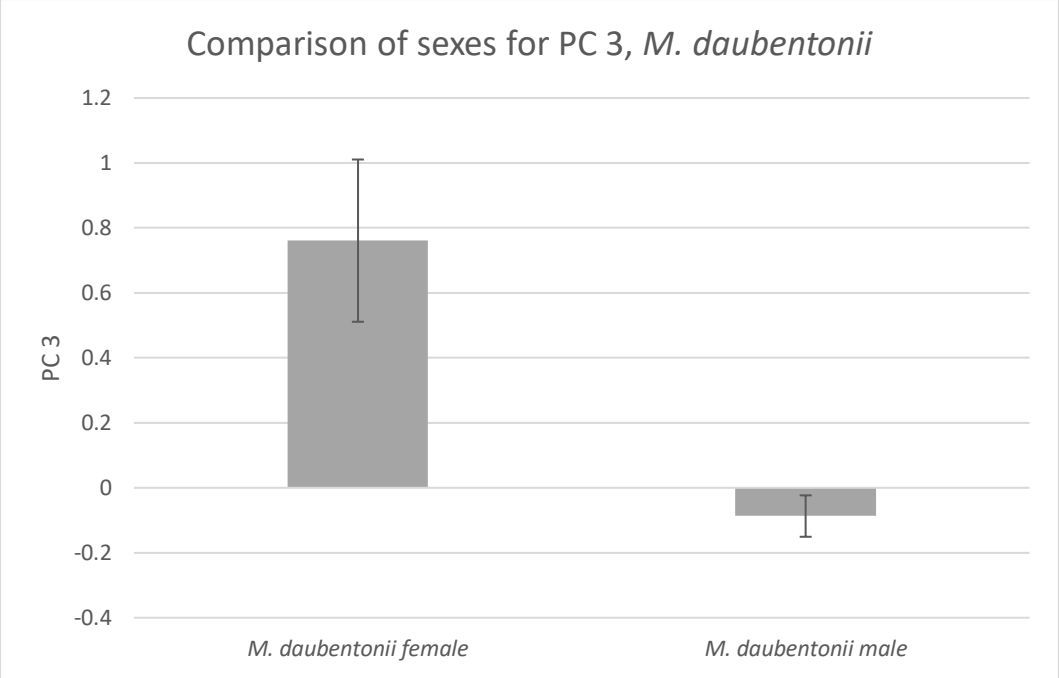


Figure 4.22 A comparison of the average scores of *M. daubentonii* males and females for PC 3.

Comparison of sexes

M. daubentonii	**Female *M. daubentonii* (n=31)**	**Male *M. daubentonii* (n=26)**	**Female *M. daubentonii* (n=31)**	**Male *M. daubentonii* (n=26)**	***U***	**p-value**
Aldosterone	0.01432	0.023666	0.018264	0.004305	194.000	.001***
Androsterone	0.002848	0.004382	0.016582	0.001229	265.000	<0.05*
Corticosterone	0.000241	0.000202	0.000426	0.000134	260.000	<0.05*
Cortisone	6.13E-05	0.000344	7.38E-05	2.7E-05	301.000	.102
Dihydrotestosterone	0.004152	0.004403	0.00796	0.002044	280.000	<0.05*
Hydrocortisone	0.000107	5.68E-05	0.000176	4.26E-05	221.000	<0.01**
Pregnenalone	0.007536	0.009047	0.012949	0.006771	338.000	.298
Progesterone	9.88E-05	8.25E-05	0.000957	4.08E-05	234.000	<0.01**
Testosterone	0.000953	0.003016	0.001655	0.000546	348.000	.378
1-dehydrotestosterone	0.000208	0.000195	0.000287	8.41E-05	255.000	<0.05*
11-deoxycorticosterone	0.000993	0.001489	0.002248	0.000523	299.000	.096
17α-hydroxyprogesterone	0.000508	0.018256	0.000946	0.000249	289.000	.068
M. nattereri	**Female *M. nattereri* (n=37)**	**Male *M. nattereri* (n=20)**	**Female *M. nattereri* (n=37)**	**Male *M. nattereri* (n=20)**	***U***	**p-value**

Aldosterone	0.014268	0.011309	0.008634	0.007557	325.000	.452
Androsterone	0.001398	0.001126	0.000968	0.000659	324.000	.442
Corticosterone	0.000272	0.000356	0.000222	0.000217	360.000	.867
Cortisone	8.22E-05	3.63E-05	3.74E-05	1.37E-05	247.500	<0.05*
Dihydrotestosterone	0.0061	0.004504	0.002926	0.002468	296.000	.216
Hydrocortisone	0.000103	9.24E-05	8.21E-05	3.77E-05	295.000	.210
Pregnenalone	0.023374	0.015808	0.014271	0.013716	319.000	.394
Progesterone	0.000382	0.000236	8.02E-05	5.01E-05	307.000	.292
Testosterone	0.000126	0.000325	3E-05	0.000216	202.000	<0.01**
1-dehydrotestosterone	9.68E-05	0.000158	4.96E-05	4.41E-05	326.000	.462
11-deoxycorticosterone	0.002223	0.003595	0.000857	0.001429	290.000	.181
17α-hydroxyprogesterone	0.00067	0.000753	0.000506	0.00026	242.000	<0.05*

Table 4.22 The results of Mann-Whitney U tests on comparisons between male and female *M. daubentonii* and *M. nattereri* for each target compound (µM/mg).

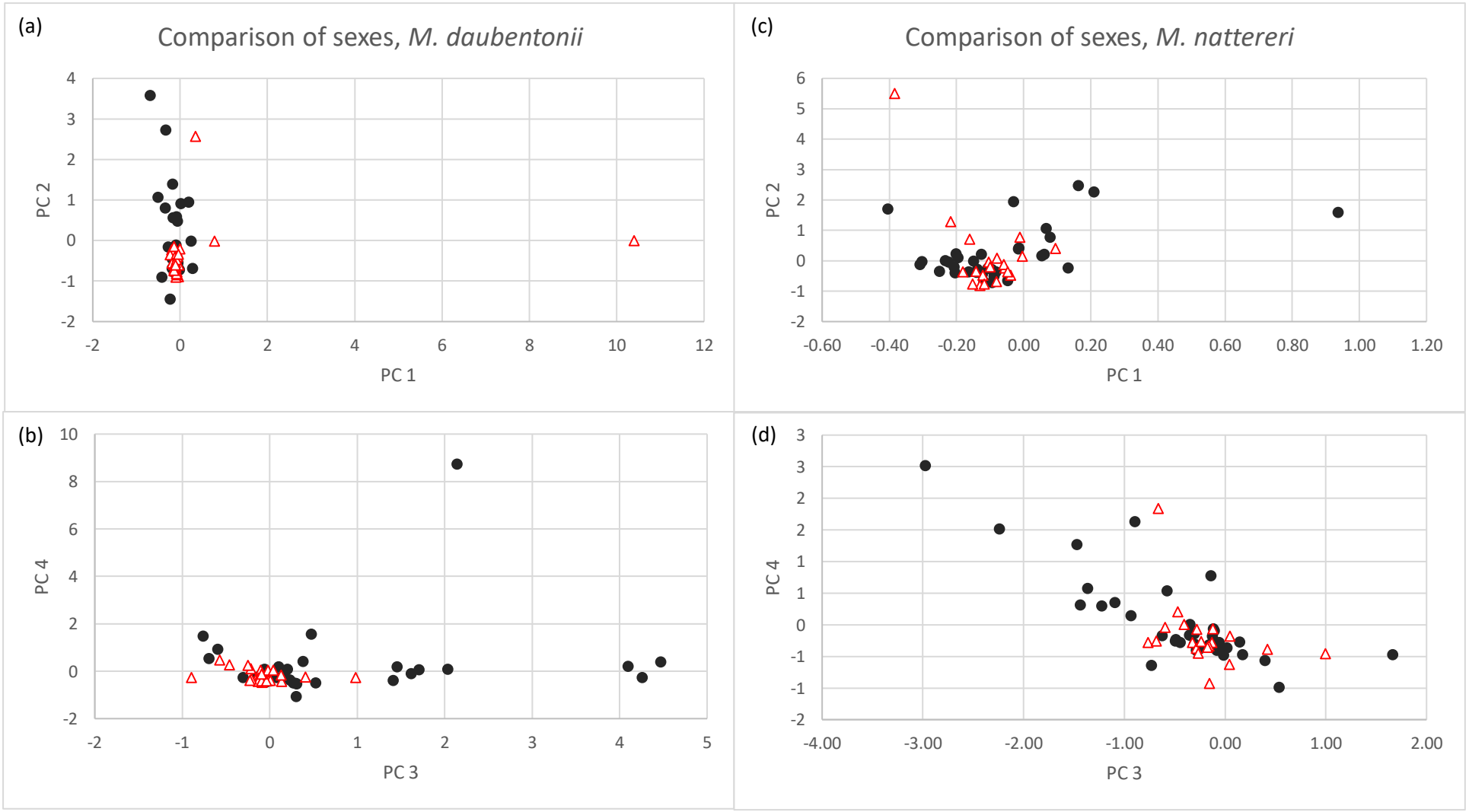


Figure 4.23 Male and female *M. daubentonii* scores for PC 1 plotted against PC 2 (a) and PC 3 against PC 4 (b) and male and female *M. nattereri* scores for PC 1 plotted against PC 2 (c) and PC 3 against PC 4 (d).

4.16.4 Comparisons between male and female *M. nattereri*

Target compounds

M. nattereri females had higher levels of one corticosteroid (cortisone) and one precursor to both sex and stress related compounds (17α-hydroxyprogesterone) than males whilst *M. nattereri* males had higher levels of one reproductive hormone (testosterone) than females (Table 4.22).

Principle components

There was no significant difference between the scores of male and female *M. nattereri* for any of the principle components (Table 4.21). Ordination plots show that most male and female scores cluster within a narrow range for each principle component, although there appears to be more variation amongst females (Figure 4.23 c and d).

4.16.5 Individual variation

Females of both species had greater variation in their principle component scores (Table 4.23). *M. daubentonii* females had more variation in three principle components (PC 2, PC 3, and PC 4). *M. nattereri* females also had greater variation in three principle components (PC 1, PC 3 and PC 4).

	PC 1	PC 2	PC 3	PC 4
M. daubentonii				
Females	0.19	1.08	1.39	1.67
Males	2.05	0.65	0.32	0.25
M. nattereri				
Females	0.21	0.84	0.78	0.71
Males	0.09	1.37	0.39	0.53

Table 4.23 Standard deviation of male and female *M. daubentonii* and *M. nattereri* for each principle component.

4.17 Discussion of differentiation among species and sex in hormone profiles

4.17.1 Species differences

There was no significant difference between species for PC 1 which accounted for most (41%) of variance. There was a significant difference between species, however, for PC 2 and PC 3. These components accounted for 20% and 9% of variance, respectively.

M. daubentonii had lower scores for PC 2 and higher scores for PC 3 than *M. nattereri*. PC 2 is composed of three glucocorticoids (corticosterone, cortisol and 11-deoxycorticosterone), one reproductive hormone and one precursor to both corticosteroids and reproductive hormones. PC 3 is composed of cortisol and corticosterone and two reproductive hormones and one precursor to both corticosteroids and reproductive hormones. Therefore, the results for PC 2 and PC 3

are someone contradictory; *M. daubentonii* had higher levels of cortisol and corticosterone in one principle component (PC 3) and lower levels in the other (PC 2).

With regards to individual compounds, *M. daubentonii* and *M. nattereri* were significantly different for reproductive hormones. *M. daubentonii* had higher levels of androsterone, testosterone and 1-dehydrotestosterone than *M. nattereri*. However, *M. nattereri* had higher levels of pregnenalone.

These differences are unlikely to be due to differences in habitat quality or differences in diet. The two species are sympatric and forage and roost in overlapping spaces (August et al. 2014). They are both insectivorous. Faecal pellet size is also unlikely to be the cause of the difference; *M. daubentonii* had larger faecal pellets (Figure 4.24) and so ought to have lower concentrations as a result of increased transit time resulting in great reabsorption from the gut or more microbial action.

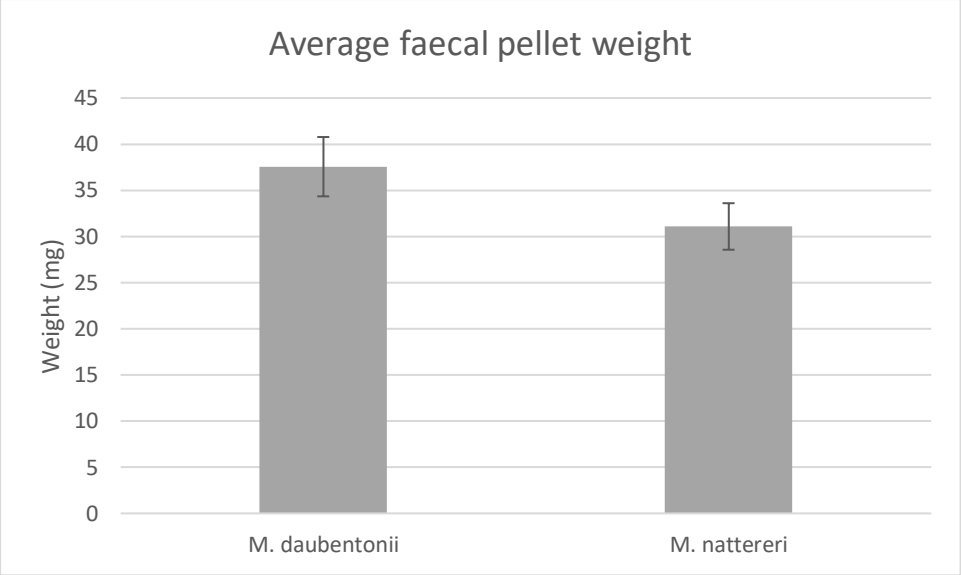


Figure 4.24 Average faecal pellet weight for *M. daubentonii* and *M. nattereri*.

4.17.2 Difference between sexes

M. daubentonii

Male *M. daubentonii* had significantly lower scores for PC 3. PC 3 is composed of cortisol and corticosterone and two reproductive hormones and one precursor to both corticosteroids and reproductive hormones.

With regards to individual compounds, *M. daubentonii* males and females had significantly different corticosteroids (females were higher for two, and males higher for 1, aldosterone a mineralocorticoid). Females had higher median levels of androsterone, progesterone, 1-dehydrotestosterone and dihydrotestosterone.

M. nattereri

There was no significant difference between males and females of *M. nattereri* for any of the principle components. *M. nattereri* females had significantly higher cortisone and 17α-hydroxyprogesterone levels than males whilst males had significantly higher testosterone levels.

In general, females of both species had higher levels of various compounds. These sex related differences could potentially be due to differences in the size of faecal pellets (Figure 4.25). Males of both species had larger faecal pellets than females. Larger faecal pellets could take longer to pass through the digestive tract which could mean more compounds are reabsorbed from the gut or microbes in the gut have longer to degrade hormones and their metabolites.

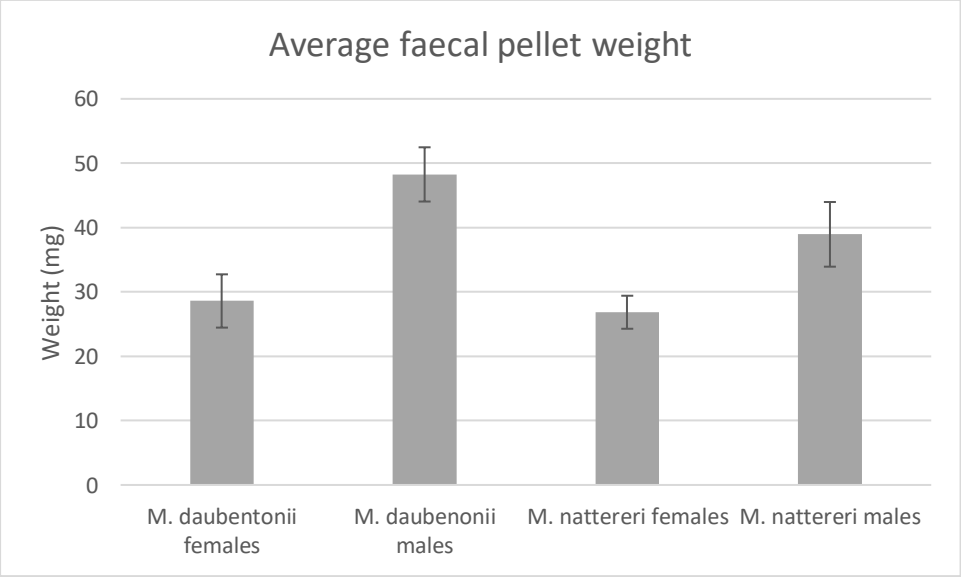


Figure 4.25 Average faecal pellet weights for female and male *M. daubentonii* and *M. nattereri*.

4.17.3 Implications for research

These results suggest that this methodology can identify predictable differences between species and sexes in bats.

Species and sexes should be studied separately with regards to investigating environmental disturbances due to significantly different corticoid and reproductive hormone levels.

Females of both species had more varied scores than males for most principle components. This suggests that when females are the focus of a study, a large number of females should be sampled in order to capture variation.

4.18 General discussion

Corticoid analysis is an increasingly popular tool for assessing the impact of anthropogenic disturbance. This pilot study sought to explore the impact of roads on corticosteroid levels in bats with a view to assessing and developing a methodology for future studies.

Corticoids were positively correlated with road density and negatively correlated with temperature. A cautious interpretation of these results suggests that glucocorticoids levels in bats are influenced by the availability of suitable foraging space and weather conditions that maximise foraging opportunities. Road density could also be associated with more frequent exposure to vehicular traffic and roadside artificial lighting.

A comparison of individuals and sexes supports the methodological decisions to focus on maternity roosts which are occupied predominantly by adult females, and

to collect and combine faecal samples from the whole roost. Future studies that explore the impact of roads on glucocorticoid levels in bats should focus on a single species and include a greater number of roosts.

Chapter 5: Discussion

Thesis pp 296-308

5.0 Discussion

5.1 Summary

Roads are a defining feature of the landscape in the UK. Almost half the UK's landcover is divided into patches of less than 5km^2. The road network is expected to expand by a further 640 kilometres of motorways and major roads by 2020. Although this is a relatively modest addition to the 50,000km of existing major roads, new roads threaten to further fragment the landscape and enhance the barrier effect.

Bats use a relatively large range for organisms of their size (3-112km^2) making them vulnerable to the impacts of roads (Bat Conservation Trust UK, 2016). Given the density of roads in the UK, bats almost certainly encounter roads frequently. If bats cross roads, they are vulnerable to collisions with vehicles. If bats avoid crossing roads, they may be largely confined to foraging within a road-defined patch. If bats are confined to foraging preferentially within road-defined patches, or if they encounter roads frequently, it is possible that bats living in landscapes fragmented by roads have heightened corticosteroid levels.

I used a three-step procedure to demonstrate that roads pose a threat to bats as a result of collisions with vehicles, that major roads act as a barrier to landscape scale movement and influence the distribution of roosts, and that the density of minor roads within the core-sustenance zone, or CSZ, is associated with heightened corticosteroid levels.

As major roads are a potential barrier to landscape scale movement, serious consideration must be given to connecting road-defined patches. Bat gantries are

ineffective and under road passages are limited in their efficacy (Berthinussen & Altringham 2012a). Green bridges should be explored as a means to connect that landscape for bats and other wildlife (Natural England 2015; Bhardwaj et al. 2017).

5.2 Discussion of key findings

5.2.1 Factors influencing road crossing behaviour and collision risk

In my thesis, I first collected evidence to show that roads affect bats. In Chapter 2, I conducted a systematic review of the literature and conducted meta-analyses to assess the threat posed by roads to bats as a result of (1) collisions with vehicles and (2) as a barrier to movement.

I found that low-flying, clutter-adapted species are both more likely to avoid crossing roads and more prone to collisions with vehicles than high flying species. Juveniles are more vulnerable to collisions than adults and there was also a significant bias towards male casualties. Collision risk and road avoidance behaviour was associated with a range of characteristics associated with the road and surrounding habitat. The presence or absence of trees and shrub layers were influential factors in determining whether bats crossed roads; the presence of either tended to increase the likelihood of road-crossing behaviour and collision risk. Bats are also more likely to cross roads at severed treelines or other linear features and where the tree canopy spans the road. There was some evidence that wider roads were more of a barrier than narrow roads and the presence of vehicles, and the volume of noise they emitted, increased the likelihood of road avoidance.

The results presented here are consistent with those reported in studies on a range of species. Roads are a barrier to movement for reptiles and amphibians (e.g. lizards, Young et al (2018); snakes, Colley et al. 2017, Robson et al. 2013; turtles, Shephard et al. 2008), mammals (Caribou, Dyer et al. 2002; cougars, Dickson et al. 2012; chipmunks, McGregor et al. 2008; grizzly bears, Northrup et al. 2012; hedgehogs, Rondinini & Doncaster 2002; squirrel gliders, van der Ree et al. 2010; white-footed mice, McGregor et al. 2008) and birds (Thinh et al. 2012).

Where studies have been able to investigate the underlying factors responsible for the barrier affect, specific characteristics have been identified similar to those in the review above in Chapter 2. These include road width (hedgehogs, Rondinini & Doncaster 2002), the width of the canopy gap (squirrel gliders, van der Ree et al. 2010) and traffic density (grizzly bears, Northrup et al. 2012). Whilst traffic noise seems to inhibit road crossing in bats, according to the studies reviewed, it does not appear to be the causal factor for road avoidance in eastern chipmunks and white-footed mice (McGregor et a. 2008).

In bats, males were disproportionately more susceptible to collisions that females. This is true also for otters (*Lutra lutra*; Philcox et al. 1999). However, there was also no effect of sex on road crossing behaviour in hedgehogs, which might suggest that there is no sex bias in terms of collision risk (Rondinini & Doncaster 2002). Whilst juvenile bats are more susceptible to collisions that adults, adult otters are more susceptible than subadults (Philcox et al. 1999). Collision risk amongst bats was associated with the presence of linear features intersecting the road. A similar effect was identified in a study on moose in Sweden (Seiler 2005).

The review included studies published prior to 2016. In 2017 an interesting study was published on bats in Brazil which appears to contradict a key finding from the review. Secco and colleagues (2017) report that collision risk was negatively associated with habitat quality, whereas the review indicates that collision risk was higher where roads bisected optimal habitats such as woodlands, or where there were ponds in the vicinity. The authors suggests that bats do not need to cross if habitat quality on the near side is sufficient. However, it should be noted that there are likely to be significant differences in the species composition and road network characteristics between this study and those conducted in the northern hemisphere, and especially Europe.

What is evident is that the response to roads varies between species, even within taxonomic groups. The causal factors responsible for the barrier affect or collision risk, also vary between species, and may depend on the species' ecology, the population's resource requirements, and the individual's life history stage.

5.2.2 Correlative study of bat distribution: The influence of major roads on the distribution of roosts

The second result of my thesis is to show that the distribution of bats is related to the presence of nearby roads. In Chapter 3 I demonstrated that the size of patches defined by major roads, and the quality of the habitat within road-defined patches, influence the distribution of roosts. Overall it appears that bats prefer to roost in larger patches with a greater area of broadleaved woodland. The importance of patch size becomes more apparent amongst smaller patches in the most fragmented artificial landscape.

As far as I have been able to ascertain, this is the first study to examine road-defined patch size. However, some key results are consistent with those from other studies. The amount of suitable habitat accessible without crossing a road is a better predictor of the presence of amphibian populations than the area of suitable habitat in the area (Eigenbrod et al. 2008). In addition, road density is negatively associated with the distribution of bats (Fagan et al. 2017) and frogs (Vos & Chardon 1998).

These results suggest that patches should be preserved (by restricting further road building), connected (possibly by green bridges, Natural England 2015; Bhardwaj et al. 2017) and improved (e.g. by planting trees). The necessity for such measures is greater amongst smaller patches in highly fragmented landscapes.

In a correlative study it is necessary to consider whether some or all of the pattern has been created by an unstudied confounding factor. It is quite probable that sampling bias exists in this dataset. Roosts that are within or close to built areas, and/or within smaller patches, are more likely to be identified than roosts in more isolated locations. In support of this hypothesis, where roosts had been identified in larger patches there was also a greater density of minor roads making them more accessible to people. Furthermore, although roosts were more likely to found in patches containing up to 20% broadleaved woodland, it declined thereafter, suggesting that roosts are more difficult to identify or regularly visit for roost counts when they are located in dense woodland. This suggests that both abundance and prevalence may have been underestimated amongst the less fragmented landscapes and therefore, the results presented here are conservative.

5.2.3 The influence of roads on the endocrinological response of bats

The third main result of my thesis is to show that baseline levels of corticosteroids in bats were associated with road density which may be due to their influence on habitat quality and foraging success. At the same time road density may be associated with an increased frequency of encounters with vehicles that trigger the emergency stress response. That corticosteroids did not correlate with proximity to roads suggests that corticosteroid levels were not related to disturbance by traffic noise.

The key implication of this work is to show that faecal endocrinology may be a promising research approach for establishing the health and, potentially, demographic status of bat populations. The technique revealed expected patterns, such as variation between sexes, which begins to authenticate it as a bioassay for other forms of variation, such as those due to environmental stressors, such as nearby roads.

Finally, it is unknown whether the corticosteroid results presented here are within the stress or allostatic overload range. Future studies should incorporate measures of stress-induced corticosteroid levels. Corticosteroids measured in blood samples taken within three minutes of capture (baseline) can be compared with corticosteroid levels measured in blood samples taken at a standardised period of time after capture (stress-induced). The strength of the stress response (i.e. the amount of change in corticosteroid levels) can then be correlated against road density. If populations living in areas with a high density of roads have a similar stress response to populations living in areas with a lower density of roads, it suggests that these populations are not chronically stressed. If on the other hand, they have a weaker

stress response it would suggest that they are chronically stressed and have either downregulated the stress response or are experiencing exhaustion.

5.3 Implications for bat conservation

Taken as a whole, the results presented in this thesis begin to establish roads as a threat to the health and sustainability of the British bat population. If, as my results tentatively suggest, fragmentation by roads has a serious detrimental impact on bats, then it is imperative that road-defined patches should be connected safely to remove barriers to the landscape scale movement of bats.

Some existing strategies are available for deployment and my work brings greater support to their use. Specifically, under-road routes (e.g. culverts) are more likely to be used than over road gantries but their efficacy is dependent on the width of the structure and is species-specific (Abbott et al 2012b; Bhardwaj et al. 2017; Ceľuch et al. 2008). Green bridges could be a more effective way to connect road-defined patches for bats and other wildlife (Natural England 2015; Bhardwaj et al. 2017).

The quality of habitat near to roosts should also be improved to better sustain bats that are trapped in small CSZs by road edges. Occupied patches tended to have a greater area of woodland than unoccupied patches. However, collisions were also more likely to be reported where roads bisected woodlands or treelines or where there was a closed tree canopy over roads (Capo et al. 2006; Iković et al. 2014; Lesiński 2007; Lesiński 2008; Lesiński et al. 2010; Medinas et al. 2012; Russell et al. 2009).

Woodland cover in England is currently less than 10%. The UK government has set a target of 12% tree cover by 2020 which is to be achieved through replanting. Care should be taken to increase woodland cover without increasing the permeability of busy roads. This could possibly be achieved by placing green bridges where roads bisect existing woodland (Natural England 2015; Bhardwaj et al. 2017).

The results presented here also indicate that building new roads, especially near maternity roosts, should be avoided. The likelihood of a roost being present within a patch defined by major roads declined as the density of minor roads within the patch increased. Juvenile bats were more vulnerable to collisions with vehicles and corticosteroid levels in maternity roosts were positively correlated with road density.

5.4 Future research

Information about roost and population size within a patch could contribute to a more detailed understanding of the impact of roads on bats.

I explored the influence of road-defined patch size on the distribution of roosts by comparing the abundance and prevalence of roosts across four artificial landscapes with differing degrees of fragmentation. However, roost size can vary greatly within and between species. We might expect that larger road-defined patches support larger roosts on average. If we combine roosts within a species and within a patch, we might find that larger patches support fewer roosts but possibly more bats.

Patch size could influence roost and population size because patch size relates to resource availability and/or because patch size influences the likelihood of crossing roads in order to access sufficient resources, and as a result influences collision risk.

If bats roosting in small patches are more likely to cross roads than bats roosting in larger patches it is possible that bat roosts are more likely to be found in larger patches as a result of unsustainable additional mortality due to collisions with vehicles.

Information on population size over time, and within patches of varying sizes, could be used to model sustainable (expected) mortality levels and to compare that with collision rates on roads bordering the patch and within the CSZ surrounding known roosts.

If patch size exerts an influence on roost and population size as well as roost location as a result of the patch size dependent availability of resources, it might be possible to compare body condition between individuals roosting in patches of varying sizes and within CSZs with varying degrees of fragmentation by roads. Controlling for roost size, bats confined to foraging within smaller patches of more fragmented CSZs could have lower weights and higher parasite loads than bats living in larger patches.

Body condition could also be correlated with the amount of time bats spend foraging each night and with corticosteroid levels. If corticosteroid levels positively correlate with the density of minor roads, and if corticosteroids influence foraging behaviour, we might expect that bats living in more fragmented CSZs forage for longer periods of time. If bats are unable to compensate by foraging for longer periods of time and experience prolonged, deleterious levels of corticosteroids in more fragmented landscapes, it is likely that they have poorer body condition which is associated with low fecundity and survival (Cayuela et al. 2017; Crino et al. 2013; Narayan et al. 2013).

5.5 Critique of the three-step risk assessment

The three-step procedure applied here could be used to examine the impact of roads on other species.

The three step procedure is as follows;

1. Review the evidence related to the potential for roads to act as a barrier to landscape scale movement.
2. Determine whether there is an effect of road-defined patch size on the distribution of bat roosts.
3. Examine whether corticosteroids correlate with road-related variables.

Here I discuss the extent to which this procedure is generalizable and how easily each of these three steps could be repeated for species other than bats.

In order to do so, there should be sufficient published research on road crossing behaviour to draw consistent conclusions as to which types of road are or are not a barrier to movement and the environmental characteristics associated with the barrier effect. In Chapter 1, I demonstrated that three rapid reviews were able to obtain as many relevant studies for red foxes, European badgers and deer, as there were for bats. If minor roads are also found to be a barrier, minor roads should also define the edge of patches for the second step of this analysis.

For some species it may be possible to investigate the influence of road-defined patch size on the presence/absence or abundance of known dens and nests (e.g. badgers' holts, the nests of birds of prey). However, for those species that do not use dens or nests, it may be preferable to investigate the influence of patch size on the abundance of individuals within a patch, or on the presence or absence of

observations of the focal species within a given patch where such data is available. Potential sources of distribution and abundance data in the UK include the Biological Records Centre (BRC) who collate species observation records from regional Wildlife Trusts, the NBN atlas (www.nbnatlas.org), or the devolved governmental departments for the environment in England, Scotland and Wales.

Habitat quality measures will depend on the species. Bats often use a wide range of habitats, or commute through them, although they often forage within or at the edges of woodland (Russ 1999). Other species may preferentially use specific habitat types found in specific regions of the UK. The Landcover map includes many habitat types that I did not quantify or that I grouped for this analysis such as bog, heath, fens and marshes. For species that are confined to a narrower range of habitat types or that have a limited known distribution, all patches that do not contain the preferred habitat types, or that are outside the known distribution of that species should be excluded before analysis in order to avoid misleading interpretations.

Probably the most important conclusion of my thesis is that faecal endocrinology in general and corticosteroid analysis in particular should be further investigated as a tool for understanding the interactions of bats with their environment – and particularly with anthropogenic stressors. Specifically, future research should attempt to validate endocrinological approaches in some important focal bat species in order to identify the most salient target hormones. As well as focusing on a single species, if one sex was found to be more inclined to avoid roads than the other, it would be preferable to focus on that sex. If possible, as well as obtaining measures of baseline corticosteroid levels, a stress-induced measure should be sampled as well as measures of body condition. By collecting faecal samples instead of handling the animals themselves, conservationists may have in future an incisive technique for

collecting information about bats without the need to handle or disturb these fascinating and valuable mammals.

Attributes of study organism	Suitable	Not suitable
Taxon	Single species	Multiple or cryptic species
Sex	Single sex	Both males and females, or unknown sex
Age	Either adults or juveniles if possible	Mixed ages, unknown age
Reproductive stage	Similar reproductive stage; e.g. non-active, pregnant, post-partum.	Mixed or unknown reproductive stage
Abundance	Common species	Rare species
Defecation	Animals that use latrines, or animals that can be captured easily and safely and defecate soon after.	Animals that defecate in inaccessible locations (e.g. cliff face nests), animals that cannot be easily or safely captured, incidental or opportunistic sample collection.

Table 4.24 The attributes of study organisms that should be considered before attempting step 3 of the risk assessment.

In order to apply the third step of this risk assessment to other species, the intended focal species should meet the criteria listed in Table 4.24. First, the study organisms must be of a single species, sex, age and reproductive stage due to predictable variation between species, sexes, ages and reproductive stages. Therefore, samples must be collected from known individuals. In addition, the need for uncontaminated samples of known age, from identified individuals, means that samples ought to be

collected soon after defecation which may require that either the animal is held by the researcher or that the researcher is within relatively close proximity to the animal. Some species may not be easily or safely handled, whilst others may not be approached easily or without causing distress. Finally, samples are more likely to be collected in suitable numbers from common species than rare species. These practical and methodological constraints make the third step of the risk assessment potentially challenging or unsuitable for some of the most at risk species; i.e. rare, shy or large mammals and birds.

The three-step risk assessment measures upstream (proximate) outcomes (behaviour, step 1; endocrinology, step 3) as well as a downstream (or ultimate) outcome (distribution, step 2). The review of studies on behavioural responses of bats to roads indicated that roads may act as a barrier; the endocrinology investigation indicated that the density of roads could be associated with stress-related hormone levels; and distribution appeared to be associated with road-defined patch size. The information obtained from these three-steps could be enhanced by incorporating studies on additional measures (Table 4.25). By assessing body condition, fitness, survival and population growth in populations living in different sized road-defined patches, or within areas with different degrees of road fragmentation, it may be possible to link the endocrinological results to the distribution results, i.e. roads cause a chronic increase in corticosteroids which cause a reduction in body condition, fitness and survival, which ultimately lead to slow population growth, or a decline, in highly fragmented areas.

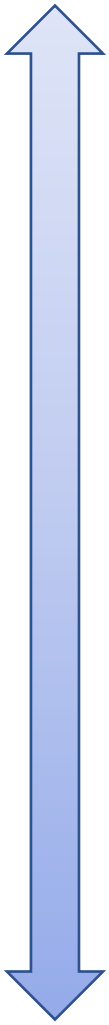
Upstream	Response variable	For example…
	Behavioural response	**Chapter 2**
	Endocrinological response	**Chapter 4**
	Body condition	Does body condition vary according to proximity to roads, road density or road-defined patch size?
	Survival	Does survivorship correlate with proximity to roads, road density or road-defined patch size?
	Fitness	Is fitness lower in populations living in areas with higher road density?
	Population growth	Does population growth rate correlate negatively with road density? It might also be possible to model the population dynamics (with information on survival and fitness) to estimate a sustainable mortality rate on roads.
	Population size	Does density decline with proximity to roads, road density or correlate with road-defined patch size?
	Distribution	**Chapter 3**
Downstream	Genetic variation	Examine differentiation between populations on either side of roads of different widths, lighting regimes or traffic densities.

Table 4.25 Potential additional approaches to investigate the impact of roads as a barrier to movement, either due to avoidance or due to collisions with vehicles.

References

Abbott IM, Butler F, Harrison S (2012a) When flyways meet highways – The relative permeability of different motorway crossing sites to functionally diverse bat species. *Landscape and Urban Planning*, 106, 293–302.

Abbott IM, Harrison S, Butler F (2012b) Clutter-adaptation of bat species predicts their use of under-motorway passageways of contrasting sizes - a natural experiment. *Journal of Zoology*, 287, 124–132.

Adams R (1996) Size-specific resource use in juvenile little brown bats*, Myotis lucifugus* (Chiroptera: *Vespertilionidae):* Is there an ontogenetic shift? *Canadian Journal of Zoology,* 74, 1204-1210.

Adams R (1997) Onset of Volancy and Foraging Patterns of Juvenile Little Brown Bats, *Myotis lucifugus. Journal of Mammalogy,* 78, 239-246.

Allen LC, Turmelle AS, Widmaier EP, Hristov NI, McCracken GF, Kunz TH (2010) Variation in Physiological Stress between Bridge- and Cave-Roosting Brazilian Free-Tailed Bats. *Conservation Biology*, 25, 374–381

Altringham JD (2011) *Bats: From Evolution to Conservation*. *Bats: From Evolution to Conservation* (2nd ed.). Oxford: Oxford University Press.

Angelier F, Meillère A, Grace JK, Trouvé C, Brischoux F (2015) No evidence for an effect of traffic noise on the development of the corticosterone stress response in an urban exploiter. *General and Comparative Endocrinology*, 232, 43-50.

Angell RL, Butlin RK, Altringham JD (2013) Sexual segregation and flexible mating patterns in temperate bats. *PloS One*, 8, e54194.

August T, Nunn M, Fensome AG, Linton DM, Mathews F (2014) Sympatric Woodland Myotis Bats Form Tight-Knit Social Groups with Exclusive Roost Home Ranges, *PLoS ONE*, 9, e112225

Balestri M, Barresi M, Campera M, Serra V, Ramanamanjato JB, et al. (2014) Habitat Degradation and Seasonality Affect Physiological Stress Levels of *Eulemur collaris* in Littoral Forest Fragments. *PLoS ONE*, 9, e107698.

Balm PHM (1999) *Stress Physiology in Animals.* Sheffield: Sheffield Academic Press Ltd

Bat Conservation Trust UK (2012) *Bats and buildings in the UK*. London: The Bat Conservation Trust

Bat Conservation Trust UK (2016) *Core Sustenance Zones : Determining zone size*. London: The Bat Conservation Trust

Bat Conservation Trust UK (2017) *The state of the UK's bats 2017: National Bat Monitoring Programme Population Trends*. London: The Bat Conservation Trust

Battersby J (2005) *UK Mammals: Species Status and Population Trends. First Report by the Tracking Mammals Partnership.* JNCC/Tracking Mammals Partnership, Peterborough.

Baxter DJM, Psyllakis JM, Gillingham MP, O'Brien EL (2006) Behavioural Response of Bats to Perceived Predation Risk While Foraging. *Ethology*, 112, 977–983.

Bender DJ, Contreras TA, Fahrig L, Ecology S, Mar N (1998) Habitat loss and population decline : A meta-analysis of the patch size effect. *Ecology*, 79**,** 517–533.

Benítez-López A, Alkemade R, Verweij PA (2010) The impacts of roads and other infrastructure on mammal and bird populations: A meta-analysis. *Biological Conservation*, 143, 1307–1316

Bennett VJ, Zurcher A (2013) When corridors collide: Road-related disturbance in commuting bats. *The Journal of Wildlife Management*, 77, 93–101.

Bernardino J, Bispo R, Costa H, Mascarenhas M (2012) Estimating bird and bat fatality at wind farms : a practical overview of estimators , their assumptions and limitations. *New Zealand Journal of Zoology*, 40, 37–41.

Berthinussen A, Altringham JD (2012a) Do bat gantries and underpasses help bats cross roads safely? *PloS One*, 7, e38775.

Berthinussen A, Altringham JD (2012b) The effect of a major road on bat activity and diversity. *Journal of Applied Ecology*, 49, 82–89.

Bhardwaj M, Soanes K, Straka TM, Lahoz-Monfort JJ, Lumsden LF, van der Ree R (2017) Differential use of highway underpasses by bats. *Biological Conservation*, 212, 22–28.

Bispo R, Bernardino J, Marques TA, Pestana D (2013) Modeling carcass removal time for avian mortality assessment in wind farms using survival analysis. *Environmental and Ecological Statistics*, 20, 147–165.

Blake D, Hutson AM, Racey PA, Rydell J, Speakman JR (1994) Use of lamplit roads by foraging bats in southern England. *Journal of Zoology*, 234, 453–462.

Border JA, Newson SE, White DCJ, Gillings S (2017) Predicting the likely impact of urbanisation on bat populations using citizen science data, a case study for Norfolk, UK. *Landscape and Urban Planning*, 162, 44–55

Boughey KL, Lake IR, Haysom KA, Dolman PM (2011) Effects of landscape-scale broadleaved woodland configuration and extent on roost location for six bat species across the UK. *Biological Conservation,* 144, 2300–2310

Bradshaw D (2007) Environmental endocrinology. *General and Comparative Endocrinology*, 152, 125–141

Brearley G, McAlpine C, Bell S, Bradley A (2012) Influence of urban edges on stress in an arboreal mammal: a case study of squirrel gliders in southeast Queensland, Australia. *Landscape Ecology,* 27, 1407-1419

Brooks TM et al (2002) Habitat Loss and Extinction in the Hotspots of Biodiversity, *Conservation Biology*, 16, 909–923

Burnham KP, Anderson D (2010) *Model Selection and Multi-Model Inference. A practical Information- Theoretic Approach*, Second Edi. Springer.

Busch DS, Hayward LS (2009) Stress in a conservation context: A discussion of glucocorticoid actions and how levels change with conservation-relevant variables. *Biological Conservation*, 142, 2844–2853

Cantarelli VI, Perez-Rueda M, Kowalewski MM, Mastromonaco GF, Ponzio MF (2017) Validation of an enzyme immunoassay and comparison of fecal cortisol metabolite levels in black and gold howler monkeys (*Alouatta caraya*) inhabiting fragmented and continuous areas of the humid Chaco region, Argentina. *American Journal of Primatology*, 79, e22625

Capo G, Chaut J, Arthur L (2006) Quatre ans d'étude de mortalité des Chiroptères sur deux kilomèters routiers proches d'un site d'hibernation. *Symbioses*, 15, 45–46.

Cayuela H, Quay L, Dumet A, Léna JP, Miaud C, Rivière V (2015) Intensive vehicle

traffic impacts morphology and endocrine stress response in a threatened amphibian. *Oryx*, 51, 182-188.

Ceľuch M, Ševčík M (2008) Road bridges as a roosts for Noctules (*Nyctalus noctula*) and other bat species in Slovakia (Chiroptera: Vespertilionidae). *Lynx*, 39, 47-54.

Chittaragi JB, Hosetti BB (2014) Road kill mortality of snakes (Squamata: Serpentes) in different land cover areas of Semi-Malnad region, Mid Western Ghats, Shimoga, India. *Current Biotica*, 8, 57–65

Clarke, G.P., White, P.C.L., Harris, S. (1998) Effects of roads on badger *Meles meles* populations in south-west England. *Biological Conservation*, 86, 117-124

Cockrem JF (2013) Individual variation in glucocorticoid stress responses in animals. *General and Comparative Endocrinology*, 181, 45-58

Coffin AW (2007) From roadkill to road ecology: A review of the ecological effects of roads. *Journal of Transport Geography*, 15, 396–406

Colley M, Lougheed SC, Otterbein K, Litzgus JD (2017) Mitigation reduces road motality of a threatened rattlesnake. *Wildlife Research*, 44, 48-59

Creel S, Fox JE, Hardy A, Sands J, Garrott B, Peterson RO (2002) Snowmobile activity and glucocorticoid stress responses in wolves and elk. *Conservation Biology,* 16, 809-814

Crino OL, Klaassen Van Oorschot B, Johnson EE, Malisch JL, Breuner CW (2011) Proximity to a high traffic road: Glucocorticoid and life history consequences for nestling white-crowned sparrows. *General and Comparative Endocrinology*, 173, 323-332

Cyr NE, Romero LM (2007) Chronic stress in free-living European starlings reduces corticosterone concentrations and reproductive success. *General and Comparative Endocrinology*, 151, 82–89

D'Amico M, Périquet S, Román J., Revilla E. (2016) Road avoidance responses determine the impact of heterogeneous road networks at a regional scale. *Journal of Applied Ecology*, 53 (1), 181-190

Dantzer B, McAdam AG, Palme R, Boutin S, Boonstra R (2011) How does diet affect fecal steroid hormone metabolite concentrations? An experimental examination in red squirrels. *General and Comparative Endocrinology*, 174, 124–131

Day J (2017) *The Impacts of street lighting on bats*. PhD. UK: Exeter.

Delehanty B, Boonstra R (2009) Impact of live trapping on stress profiles of Richardson's ground squirrel (*Spermophilus richardsonii*). *General and Comparative Endocrinology*, 160, 176–182

Department for Transport (2014) *Road Investment Strategy: Overview*. London.

Department for Transport (2017a) *Road traffic estimates 2016*. London.

Department for Transport (2017b) *Road Lengths in Great Britain 2016*. London.

Dominoni D, Quetting M, Partecke J (2013) Artificial light at night advances avian reproductive physiology. *Proceedings of the Royal Society B: Biological sciences*, 280, 20123017.

Downs NC, Racey P (2006) The use by bats of habitat features in mixed farmland in Scotland. *Acta Chiropterologica*, 8, 169–185.

Dulac J (2013) *Global land transport infrastructure requirements: Estimating road and railway infrastructure capacity and costs to 2050*. International Energy Agency, France.

Eigenbrod F, Hecnar SJ, Fahrig L (2007) Accessible habitat: an improved measure of the effects of habitat loss and roads on wildlife populations. *Landscape Ecology*, 23, 159–168

Ellis EC (2011) Anthropogenic transformation of the terrestrial biosphere. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences*, 369, 1010–1035

Ellis EC, Ramankutty N (2008) Putting people in the map: Anthropogenic biomes of the world. *Frontiers in Ecology and the Environment*, 6, 439–447

Ellis RD, McWhorter TJ (2012) Integrating landscape ecology and conservation physiology. *Landscape Ecology in Review*, 2, 1-12

Erritzoe J, Mazgajski TD, Rejt Ł (2003) Bird casualties on European roads — A review. *Acta Ornithologica*, 38, 77–93.

ESRI 2014. ArcGIS Desktop: Release 10. Redlands, CA: Environmental Systems Research Institute.

European Commission (2013) *Eurostat*. Available from: http://ec.europa.eu/eurostat/web/transport/data/database (accessed July 15, 2015)

European Commission (2014) *Infrastructure - TEN-T - Connecting Europe*. Available from: http://ec.europa.eu/transport/themes/infrastructure/index_en.htm

(accessed July 15, 2015)

European Commission (2017) *EU Funding for TEN-T*. Available from https://ec.europa.eu/transport/themes/infrastructure/ten-t-guidelines/project-funding_en (accessed September 14, 2017).

Fagan KE, Willcox EV, Tran LT, Bernard RF, Stiver WH (2018) Roost selection by bats in buildings, Great Smoky Mountains National Park. *The Journal of Wildlife Management*, 82(2), 424-434

Fahrig L, Pedlar JH, Pope SE, Taylor PD, Wegner JF (1995) Effect of road traffic on amphibian density. *Biological Conservation*, 73, 177–182.

Fahrig L, Rytwinski T (2009) Effects of roads on animal abundance: an empirical review and synthesis. *Ecology and Society*, 14, article 21

Fensome AG, Mathews F (2016) Roads and bats: A meta-analysis and review of the evidence on vehicle collisions and barrier effects. *Mammal Review*, 46, 311-323

Fensome AG (2013, Autumn) "Do human-altered envionments stress bats? Complimentary approaches to study bats: swimming upstream". *Mammal News,* 167, 12-13

Fletcher QE, Dantzer B, Boonstra R (2015) The impact of reproduction on the stress axis of free-living male northern red backed voles (*Myodes rutilus*). *General and Comparative Endocrinology*, 224, 136–147

Fonken LK, Finy MS, Walton JC, Weil ZM, Workman JL, Ross J, Nelson RJ (2009) Influence of light at night on murine anxiety- and depressive-like responses. *Behavioural Brain Research*, 205, 349–354

Forman RTT, Alexander LE (1998) Roads and their major ecological effects. *Annual*

Review of Ecology and Systematics, 29, 207–231

Gaisler J, Øehák Z, Bartonièka T (2009) Bat casualties by road traffic (Brno-Vienna). *Acta Theriologica*, 54, 147–155.

Galantinho A, Eufrázio S, Silva C, Carvalho F, Alpizar-Jara R, Mira A (2017) Road effects on demographic traits of small mammal populations. *European Journal of Wildlife Research*, 63, article 22

Gaston KJ & Bennie J (2014) Demographic effects of artificial nighttime lighting on animal populations. *Environmental Reviews*, 22, 323-330

Glista DJ, DeVault TL, DeWoody JA (2008) Vertebrate road mortality predominantly impacts amphibians. *Herpetological Conservation and Biology*, 3, 77–87.

Gobush KS, Mutayoba BM, Wasser SK (2008) Long-term impacts of poaching on relatedness, stress physiology, and reproductive output of adult female African Elephants. *Conservation Biology*, 22, 1590-1599

Gonzalez-Prieto S, Villarino A, Freán M (1993) Mortalidade de vertebrados por atropello en una carretera nacional del NO de Espana. *Ecologia*, *7*, 375–389.

Goyman W (2012) On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. *Methods in Ecology and Evolution*, 3, 757-765

Grilo C, Bissonette JA, Santos-Reis M (2015) No evidence of a threshold in traffic volume affecting road-kill mortality at a large spatio-temporal scale. *Environmental Impact Assessment Review*, 55, 54-58

Haddad NM et al. (2015) Habitat fragmentation and its lasting impact on Earth's ecosystems. *Science Advances*, 1, e1500052–e1500052.

Haensel J, Rackow W (1996) Fledermäuse als Verkehrs- opfer — ein neuer Report. *Nyctalus*, *NF*(6), 29–47.

Hale JD, Fairbrass AJ, Matthews F, Thomas J, Davies G, Sadler JP (2015) The ecological impact of city lighting scenarios: exploring gap crossing thresholds for urban bats. *Global Change Biology*, *21*, 2467–2478.

Hale JD, Fairbrass AJ, Matthews TJ, Sadler JP (2012) Habitat composition and connectivity predicts bat presence and activity at foraging sites in a large uk conurbation. *PLoS ONE*, 7, e33300

Harris S, Morris P, Wray S, Yalden D (1995) *A review of British mammals: population estimates and conservation status of British*. JNCC, Peterborough.

Heigl F, Horvath K, Laaha G, Zaller JG (2017) Amphibian and reptile road-kills on tertiary roads in relation to landscape structure: using a citizen science approach with open-access land cover data. BMC Ecology, 17, article 24.

Hels T, Buchwald E (2001) The effect of road kills on amphibian populations. *Biological Conservation*, 99, 331–340.

Holderegger R, Di Giulio M (2010) The genetic effects of roads: A review of empirical evidence. *Basic and Applied Ecology*, 11, 522–531.

House of Commons Hansard (2003) 412.

Huso MMP (2011) An estimator of wildlife fatality from observed carcasses. *Environmetrics*, 22, 318–329.

Ibisch PL, Hoffmann MT, Kreft S, Pe G, Kati V, Biber-freudenberger L, Dellasala DA, Vale MM, Hobson PR, Selva N (2017) A gobal map of roadless areas and their conservation status. *Science*, 354, 349–352.

IUCN (2015) The IUCN Red List of Threatened Species.Version 2015.2.

Jaeger JAG, Bowman J, Brennan J, Fahrig L, Bert D, Bouchard J, Charbonneau N, Frank K, Gruber B, von Toschanowitz KT (2005) Predicting when animal populations are at risk from roads: an interactive model of road avoidance behavior. *Ecological Modelling*, 185, 329–348.

Jakubas D, Ryś M, Lazarus M. (2018) Factors affecting wildlife-vehicle collision on the expressway in a suburban area in northern Poland. *North-Western Journal of Zoology*, 14(1), 107-116

Iković V, Đurović M, Presetnik P (2014) First data on bat traffic casualties in Montenegro. *Vespertilio*, *17*, 89–94.

Kämmerle JL, Brieger F, Kröschel M, Hagen R, Storch I, Suchant R. (2017) Temporal patterns in road crossing behaviour in roe deer (*Capreolus capreolus*) at sites with wildlife warning reflectors. *PLoS ONE*, 12(9), e0184761

Karlson M, Mörtberg U (2015) A spatial ecological assessment of fragmentation and disturbance effects of the Swedish road network. *Landscape and Urban Planning*, 134, 53–65.

Kato Y, Amaike Y, Tomioka T, Oishi T, Uraguchi K, Masuda R. (2017) Population genetic structure of the urban fox in Sapporo, northern Japan. *Journal of Zoology*, 301(2), 118-124

Keay JM, Singh J, Gaunt MC, Kaur T (2006) Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: A literature review. *American Association of Zoo Veterinarians*, 37, 234-244

Kelm DH, Popa-Lisseanu AG, Dehnhard M, Ibáñez C (2016) Non-invasive monitoring

of stress hormones in the bat *Eptesicus isabellinus* – Do fecal glucocorticoid metabolite concentrations correlate with survival? *General and Comparative Endocrinology*, 226, 27–35

Kelt DA, Vuren D Van (1999) Energetic constraints and the relationship between body size and home range area in mammals. *Ecology*, 80, 337–340.

Kerth G, Melber M (2009) Species-specific barrier effects of a motorway on the habitat use of two threatened forest-living bat species. *Biological Conservation*, 142, 270–279.

Kiefer A, Merz H, Rackow W, Roer H, Schlegel D (1995) Bats as traffic casualties in Germany. *Myotis*, 32, 215–220.

Kirkpatrick L, Maher SJ, Lopez Z, Lintott PR, Bailey SA, Dent D, Park KJ (2017) Bat use of commercial coniferous plantations at multiple spatial scales: Management and conservation implications. *Biological Conservation*, 206, 1–10.

Kitzes J, Merenlender A (2014) Large roads reduce bat activity across multiple species. *PLoS ONE*, 9, e96341.

Klose SM, Smith CL, Denzel AJ, Kalko EKV (2006) Reproduction elevates the corticosterone stress response in common fruit bats. *Journal of Comparative Physiology A*, 192, 341-350

Kociolek A V., Clevenger AP, St. Clair CC, Proppe DS (2011) Effects of road networks on bird populations. *Conservation Biology*, 25, 241–249.

Kunz TH, Fenton MB (2003) *Bat Ecology*. Chicago: The University of Chicago Press.

Kušta T, Keken Z, Ježek M, Holá M, Šmíd P. (2017) The effect of traffic intensity and animal activity on probability of ungulate-vehicle collisions in the Czech Republic.

Safety Science, 91, 105-113

Landys MM, Ramenofsky M, Wingfield JC (2006) Actions of glucocorticoids at a seasonal baseline as compared to stress-related levels in the regulation of periodic life processes. *General and Comparative Endocrinology*, 148, 132-149

Langbein J (2011) *Monitoring reported deer road casualties and related accidents in England to 2010. Final report to the Highways Agency*. The Deer Initiative, Wrexham, UK

Lawton, J.H., Brotherton, P.N.M., Brown, V.K., Elphick, C., Fitter, A.H., Forshaw, J., Haddow, R.W., Hilborne, S., Leafe, R.N., Mace, G.M., Southgate, M.P., Sutherland, W.J., Tew, T.E., Varley, J., & Wynne GR (2010) *Making space for nature: A review of England's wildlife sites and ecological network.* Report to Defra:107.

Lesiński G (2007) Bat road casualties and factors determining their number. *Mammalia*, 71, 138–142.

Lesiński G (2008) Linear landscape elements and bat casualties on roads — an example. *Annales Zoologici Fennici*, 45, 277–280.

Lesiński G, Sikora A, Olszewski A (2010) Bat casualties on a road crossing a mosaic landscape. *European Journal of Wildlife Research*, 57, 217–223.

Lewanzik D, Kelm DH, Greiner S, Dehnhard M, Voigt CC (2012) Ecological correlates of cortisol levels in two bat species with contrasting feeding habits. *General and Comparative Endocrinology*, 177, 104–112

Lian X, Zhang T, Cao Y, Su J, Thirgood S (2011) Road proximity and traffic flow perceived as potential predation risks: evidence from the Tibetan antelope in the

Kekexili National Nature Reserve, China. *Wildlife Research*, 38, 141-146

Lintott PR, Bunnefeld N, Fuentes-montemayor E, Minderman J, Mayhew RJ, Olley L, Park KJ (2014) City life makes females fussy : sex differences in habitat use of temperate bats in urban areas. *Royal Society Open Science*, 1, 1–8.

Longcore T & Rich C (2004) Ecological Light Pollution*. Frontiers in Ecology and the Environment,* 2, 191-198

Lunde ET, Bech C, Fyumagwa RD, Jackson CR, Røskaft E (2016) Assessing the effect of roads on impala (*Aepyceros melampus*) stress levels using faecal glucocorticoid metabolites. *African Journal of Ecology*, 54, 434-441

Mackie IJ, Racey PA (2007) Habitat use varies with reproductive state in noctule bats (Nyctalus noctula): Implications for conservation. *Biological Conservation*, 140, 70–77.

Mathews F, Roche N, Aughney T, Day J, Baker J, Langton S (2015) Barriers and benefits: implications of artificial night lighting for the distribution of common bats in Britain and Ireland. *Philosophical Transaction of the Royal Society B*, 370, 1-13.

McEwen BS, Wingfield JC (2003) The concept of allostasis in biology and biomedicine. *Hormones and Behavior*, 43, 2-15

McGregor RL, Bender DJ, Fahrig L (2008) Do small mammals avoid roads because of the traffic? *Journal of Appled Ecology*, 45, 117-123

Medinas D, Marques JT, Mira A (2012) Assessing road effects on bats: the role of landscape, road features, and bat activity on road-kills. *Ecological Research*, 28, 227–237.

Meisingset EL, Loe LE, Brekkum O, Van Moorter B, Mysterud A. (2013) Red deer habitat selection and movements in relation to roads. *Journal of Wildlife Management,* 77(1), 181-191

Millspaugh JJ, Washburn BE (2004) Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *General and Comparative Endocrinology*, 138, 189–199

Morton D, Rowland C, Wood C, Meek L, Marston C, Smith G, Wadsworth R, Simpson IC (2011) Countryside Survey: Final Report for LCM2007 – the new UK Land Cover Map. Countryside Survey Technical Report No 11/07 NERC/Centre for Ecology & Hydrology:112 (CEH Project Number: C03259).

Möstl E, Palme R (2002) Hormones as indicators of stress. *Domestic Animal Endocrinology*, 23, 67-74

Müllner A, Linsenmair KE, Wikelski M (2004) Exposure to ecotourism reduces survival and affects stress response in hoatzin chicks (*Opisthocomus hoazin*). *Biological Conservation*, 118, 549–558

Myczko Ł, Sparks TH, Skórka P, Rosin ZM, Kwieciński Z, Górecki MT, Tryjanowski P (2017) Effects of local roads and car traffic on the occurrence pattern and foraging behaviour of bats. *Transportation Research Part D*, 56, 222-228.

Narayan EJ, Cockrem JF, Hero JM (2013) Repeatability of baseline corticosterone and short-term corticosterone stress responses, and their correlation with testosterone and body condition in a terrestrial breeding anuran (*Platymantis vitiana*). *Comparative Biochemistry and Physiology, Part A*, 165, 304–312

Natural England (2015) *Green Bridges-A literature review. Natual England Commissioned Report* NECR181.

Navara KJ & Nelson RJ (2007) The dark side of light at night: physiological, epidemiological, and ecological consequences. *Journal of Pineal Research*, 43, 215-224

Northrup JM, Pitt J, Muhly TB, Stenhouse GB, Musiani M, Boyce MS (2012) Vehicle traffic shapes grizzly bear behaviour on a multiple use landscape. *Journal of Applied Ecology*, 49, 1159-1167

Ogden LJE (1996) *Collision course: the hazards of lighted structures and windows to migrating birds*. Toronto, Canada: World Wildlife Fund Canada and Fatal Light Awareness Program.

Oprea M, Mendes P, Vieira TB, Ditchfield AD (2009) Do wooded streets provide connectivity for bats in an urban landscape? Biodiversity and Conservation, 18, 2361–2371.

Ouyang JQ, de Jong M, Hau M, Visser ME, van Grunsven RHA, Spoelstra K (2015) Stressful colours: corticosterone concentrations in a free-living songbird vary with the spectral composition of experimental illumination. *Biology Letters*, 11, 20150517.

Owen DAS, Carter ET, Holding ML, Islam K, Moore IT (2014) Roads are associated with blunted stress response in a North American pit viper. *General and Comparative Endocrinology*, 202, 87-92.

Parsons KN, Jones G, Davidson-Watts I, Greenaway F (2003) Swarming of bats at underground sites in Britain - Implications for conservation. *Biological Conservation*, 111, 63–70.

Philcox CK, Grogan AL, MacDonald DW (1999) Patterns of otter *Lutra lutra* road

mortality in Britain. *Journal of Applied Ecology*, 36, 748-762

Pickett S (1989) Space-for-time substitution as an alternative to long-term studies. *Long-Term Studies in Ecology: Approaches and Alternatives*, ed Likens GE (Springer, New York), pp 110‑135.

Powers LV, Kandarian SC, Kunz TH (1991) Ontogeny of flight in the little brown bat, Myotis lucifugus: behavior, morphology, and muscle histochemistry. *Journal of Comparative Physiology*, 168, 675-685.

R Core Team (2014) *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna.

Rackow W, Harz O, Schlegel D (1994) Fledermäuse (Chiroptera) als Verkehrsopfer in Niedersachsen. *Nyctalus*, 5, 11–18.

Ransome R (1990) *The Natural History of Hibernating Bats*. Bromley, Christopher Helm (Publishers) Ltd

Reeder DM, Kramer KM (2005) Stress in free-ranging mammals: Integrating physioogy, ecology, and natural history. *Journal of Mammalogy*, 86, 225-235

Reeder DM, Kosteczko NS, Kunz TH, Widmaier EP (2004a) Changes in baseline and stress-induced glucocorticoid levels during the active period in free-ranging male and female little brown myotis, *Myotis lucifugus* (Chiroptera: Vespertilionidae). *General and Comparative Endocrinology*, 136, 260–269

Reeder DM, Kunz TH, Widmaier EP (2004b) Baseline and Stress-Induced Glucocorticoids During Reproduction in the Variable Flying Fox, *Pteropus hypomelanus* (Chiroptera: Pteropodidae). *Journal of Experimental Zoology*, 301A, 682-690

Rimbach R, Link A, Heistermann M, Gómez-Posada C, Galvis N, Heymann EW (2013) Effects of logging, hunting, and forest fragment size on physiological stress levels of two sympatric ateline primates in Colombia. *Conservation Physiology,* 1, doi:10.1093/conphys/cot031.

Robert KA, Lesku JA, Partecke J, Chambers B (2015) Artificial light at night desynchronizes strictly seasonal reproduction in a wild mammal. *Proceedings of the Royal Society B: Biological sciences*, 282, 20151745

Robson LE, Blouin-Demers G (2013) Eastern Hognose Snakes (Heterodon platirhinos) Avoid Crossing Paved Roads, but Not Unpaved Roads. *Copeia*, 3, 507-511

Romero LM (2002) Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. *General and Comparative Endocrinology*, 128, 1-24

Romero M (2004) Physiological stress in ecology: lessons from biomedical research. *Trends in Ecology and Evolution*, 19, 249-255

Rondinini C, Doncaster CP (2002) Roads as a barrier for hedgehogs. *Functional Ecology*, 16, 504-509

Russ J (1999) *The Bats of Britain and Ireland: Echolocation Calls, Sound Analysis and Species Identification*. Alana Books, Powys.

Russ A, Reitemeier S, Weismann A, Gottschalk J, Einspanier A, Klenke R (2015) Seasonal and urban effects on the endocrinology of a wild passerine. *Ecology and Evolution*, 5, 5698–5710

Russell A, Butchkoski C, Saidak L, McCracken, G. (2009) Road-killed bats, highway design, and the commuting ecology of bats. *Endangered Species Research*, 8, 49–60.

Russo D, Ancillotto L (2015) Sensitivity of bats to urbanization: A review. *Mammalian Biology*, 80, 205–212.

Rydell J (1992) Exploitation of Insects around Streetlamps by Bats in Sweden. *Functional Ecology*, 6, 744-750.

Salmon M, Tolbert MG, Painter DP, Goff M, Reiners R (1995) Behavior of logger-head sea turtles on an urban beach. II. Hatchling orientation. *Journal of Herpetology*, 29, 568–76

Santos SM, Carvalho F, Mira A (2011) How long do the dead survive on the road? Carcass persistence probability and implications for road-kill monitoring surveys. *PloS One*, 6, e25383.

Sapolsky RM, Romero LM, Munck AU (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews*, 21, 55-89

Schaub A, Ostwald J, Siemers BM (2008) Foraging bats avoid noise. *The Journal of Experimental Biology*, 211, 3174–3180.

Schloss CA, Nunez TA, Lawler JJ (2012) Dispersal will limit ability of mammals to track climate change in the Western Hemisphere. *Proceedings of the National Academy of Sciences*, 109, 8606–8611.

Schofield HW, Mitchell-Jones AJ (2011) *The Bats of Britain and Ireland* (Fourth Edi). Vincent Wildlife Trust, Ledbury.

Schorcht W, Bontadina F, Schaub M (2009) Variation of adult survival drives population dynamics in a migrating forest bat. *Journal of Animal Ecology*, 78, 1182–1190.

Schwarzer G (2015) *meta: General Package for Meta-Analysi*s. R package version 4.1-0.

Secco H, Gomes LA, Lemos H, Mayer F, Machado T, Guerreiro M, Gregorin R (2017) Road and landscape features that affect bat roadkills in Southeastern Brazil. *Oecologia Australis*, 21, 323-336

Seiler A (2005) Predicting locations of moose-vehicle collision in Sweden. *Journal of Applied Ecology*, 42, 371-382

Seltmann A, Czirják GÁ, Courtiol A, Bernard H, Struebig MJ, Voigt CC (2017) Habitat disturbance results in chronic stress and impaired health status in forest-dwelling paleotropical bats. *Conservation Physiology*, 5, doi:10.1093/conphys/cox020.

Senior P, Butlin RK, Altringham JD (2005) Sex and segregation in temperate bats. *Proceedings of the Royal Society B: Biological sciences*, 272, 2467–73.

Shepard DB, Kuhns AR, Dreslik MJ, Phillips CA (2008) Roads as barrier to animals movement in fragmented landscapes. *Animal Conservation*, 11, 288-296.

Sheriff MJ, Dantzer B, Delehanty B, Palme R, Boonstra R (2011) Measuring stress in wildlife: techniques for quantifying glucocorticoids. *Oecologia*, 166, 869–887

Shyama Prasad Rao R, Saptha Girish MK (2007) Road kills: Assessing insect casualties using flagship taxon. *Current Science*, 92, 830–837.

Siemers BM, Schaub A (2011) Hunting at the highway: traffic noise reduces foraging efficiency in acoustic predators. *Proceedings of the Royal Society B: Biological sciences*, 278, 1646–1652.

Sosa R, Schalk CM (2016) Seasonal activity and species habitat guilds influence road-

kill patterns of neotropical snakes. *Tropical Conservation Science*, 9, 1-12

Spellerberg IF (1998) Ecological effects of roads and traffic : a literature review. *Global Ecology and Biogeography Letters*, 7, 317–333.

Stone EL, Jones G, Harris S (2009) Street lighting disturbs commuting bats. *Current Biology*, 19, 1123–1127.

Stone EL, Harris S, Jones G (2015) Impacts of artificial lighting on bats: a review of challenges and solutions. *Mammalian Biology*, 80, 213–219

Strasser EH, Heath JA (2013) Reproductive failure of a human-tolerant species, the American kestrel, is associated with stress and human disturbance. *Journal of Applied Ecology*, 50, 912-919.

Telleria JL, Diaz JA, Perez-Tris J, de Juana E, de la Hera I, Iraeta P, Salvador A, Santos T (2011) Barrier effects on vertebrate distribution caused by a motorway crossing through fragmented forest landscape. *Animal biodiversity and Conservation*, 34, 331-340

Tempel DJ, Gutiérrez RJ (2004) Factors related to fecal corticosterone levels in California Spotted Owls: Implications for assessing chronic stress. *Conservation Biology*, 18, 538-547.

Thinh VT, Doherty PF, Bui TH, Huyvaert KP (2012) Road crossing by birds in a tropical forest in Northern Vietnam. *Condor*, 11, 639-644

Torres A, Jaeger JAG, Alonso JC (2016) Assessing large-scale wildlife responses to human infrastructure development. *Proceedings of the National Academy of Sciences*, 113, 8472–8477.

Trombulak SC, Frissell C (2001) Review of ecological effects of roads on terrestrial

and aquatic communities. *Conservation Biology*, 14, 18–30.

Vitousek MN, Jenkins BR, Safran RJ (2014) Stress and success: Individual differences in the glucocorticoid stress response predict behavior and reproductive success under high predation risk. *Hormones and Behavior*, 66, 812–819

Voigt CC, Schneeberger K, Voigt-Heucke SL, Lewanzik D (2011) Rain increases the energy cost of bat flight. *Biology Letters*, 7, 793-795

Voigt CC, Kingston T (2015) *Bats in the anthropocene: Conservation of bats in a changing world*. Springer International Publishing

Vos CC, Chardon JP (1998) Effects of habitat fragmentation and road density on the distribution pattern of the moor frog *Rana arvalis*. *Journal of Applied Ecology*, 35, 44-56

Washburn BE, Millspaugh JJ (2002) Effects of simulated environmental conditions on glucocorticoid metabolite measurements in white-tailed deer feces. *General and Comparative Endocrinology*, 127, 217–222

Walsh AL, Harris S (1996) Foraging habitat preferences of Vespertilionid bats in Britain. *Journal of Applied Ecology*, 33, 508–518.

Wasser AK, Bevis K, King G, Hanson E (1997) Noninvasive physiological measures of disturbance in the Northern Spotted Owl. *Conservation Biology*, 11, 1019-1022

Web of Science (2018) Web of Science Service for UK Education- Home Page, Available from: http://wok.mimas.ac.uk/ Accessed: 11 July 2018.

Widmaier EP, Kunz TH (1993) Basal, diurnal, and stress-induced levels of glucose and glucocorticoids in captive bats. *The Journal of Experimental Zoology*, 265, 533-540

Wikelski M, Cooke SJ (2006) Conservation Physiology. *Trends in Ecology and Evolution*, 21, 38-46

Winn J, Tierney M, Heathwaite AL, Jones, L, Paterson J, Simpson L, Thomson A, Turley C (2011) T*he Drivers of change in UK ecosystems and ecosystem services.* In*: The UK National Ecosystem Assessment Technical Report*. UK National Ecosystem Assessment, UNEP-WCMC, Cambridge.

Young ME, Ryberg WA, Fitzgerald LA, Hibbitts, TJ (2018) Fragmentation alters home range and movements of the Dunes Sagebrush Lizard (*Sceloporus arenicolus*). *Canadian Journal of Zoology*, 96, 905-912

Zurcher AA, Sparks DW, Bennett VJ (2010) Why the Bat Did Not Cross the Road? *Acta Chiropterologica*, 12, 337–340.

Appendices

Appendix 1.1 Systematic review of the literature pertaining to bats and roads

A total of 400 studies were assessed for relevance in two databases (Google Scholar and Web of Science). Studies were selected if they investigated the impact of roads in relation to bat ecology and behaviour. *In Google Scholar*, the first two hundred titles retrieved with the search terms “roads AND bats OR Chiroptera” were checked for relevant studies. In *Web of Science*, the first two hundred titles retrieved with the search terms “roads AND bats OR Chiroptera” were checked for relevant studies.

References for Figure 1.3

Abbott IM, Butler F, Harrison S (2012a) When flyways meet highways – The relative permeability of different motorway crossing sites to functionally diverse bat species. *Landscape and Urban Planning*, 106, 293 -302.

Abbott IM, Harrison S, Butler F (2012b) Clutter-adaptation of bat species predicts their use of under-motorway passageways of contrasting sizes - a natural experiment. *Journal of Zoology*, 287, 124–132.

Allen LC, Turmelle AS, Widmeir EP, Hristov NI, McCracken GF, Kunz TH (2010) Variation in physiological stress between bridge- and cave- roosting Brazilian free-tailed bats. *Conservation Biology*, 25, 374-381.

Bennett FM, Loeb SC, Bunch MS, Bowerman WW (2008) Use and selection of bridges as day roosts by Rafinesque’s big-eared bats. *American Midland Naturalist*, 160, 386-399.

Bennett VJ, Spark DW, Zollner PA (2013a) Modeling the indirect effects of road networks on the foraging activities of bats. *Landscape Ecology*, 28, 979-991.

Bennett VJ, Zurcher A (2013b) When corridors collide: Road-related disturbance in commuting bats. *The Journal of Wildlife Management*, 77, 93–101

Berthinussen A, Altringham JD (2012a) Do bat gantries and underpasses help bats cross roads safely? *PloS One*, 7, e38775.

Berthinussen A, Altringham JD (2012b) The effect of a major road on bat activity and diversity. *Journal of Applied Ecology*, 49, 82–89.

Bhardwaj M, Soanes K, Straka TM, Lahoz-Monfort JJ, Lumsden LF, van der Ree R (2017) Differential use of highway underpasses by bats. *Biological Conservation*, 212, 22-28.

Blake D, Hutson AM, Racey PA, Rydell J, Speakman JR (1994) Use of lamplit roads by foraging bats in southern England. *Journal of Zoology*, 234, 453–462.

Bonsen G, Law B, Ramp D (2015) Foraging strategies determine the effect of traffic noise on bats. *Acta Chiropterologica*, 17, 347-357.

Capo G, Chaut J, Arthur L (2006) Quatre ans d'étude de mortalité des Chiroptères sur deux kilomèters routiers proches d'un site d'hibernation. *Symbioses*, 15, 45–46.

Ceľuch M, Ševčík M (2008) Road bridges as a roosts for Noctules (*Nyctalus noctula*) and other bat species in Slovakia (Chiroptera: Vespertilionidae). *Lynx*, 39, 47-54.

Ceron K, Savariz Bôlla DA, Lidorio de Mattia D, Carvalho F, Zocches JJ (2017) Roadkilled bats (Mammalia: Chiroptera) in two highways of Santa Catarina state, Southern Brazil. *Oecologia Australis, 21, 207-212.*

Gaisler J, Øehák Z, Bartonièka T (2009) Bat casualties by road traffic (Brno-Vienna), *Acta Theriologica*, 54, 147–155.

Gonzalez-Prieto S, Villarino A, Freán M (1993) Mortalidade de vertebrados por atropello en una carretera nacional del NO de Espana. *Ecologia*, *7*, 375–389.

Haensel J, Rackow W (1996) Fledermäuse als Verkehrs- opfer — ein neuer Report. *Nyctalus*, *NF*(6), 29–47.

Iković V, Đurović M, Presetnik P (2014) First data on bat traffic casualties in Montenegro. *Vespertilio*, *17*, 89–94.

Kerth G, Melber M (2009) Species-specific barrier effects of a motorway on the habitat use of two threatened forest-living bat species. *Biological Conservation*, 142, 270–279.

Kiefer A, Merz H, Rackow W, Roer H, Schlegel D (1995) Bats as traffic casualties in Germany. *Myotis*, 32, 215–220.

Kitzes J, Merenlender A (2014) Large roads reduce bat activity across multiple species. *PLoS ONE*, 9, e96341.

Lesiński G (2007) Bat road casualties and factors determining their number. *Mammalia*, 71, 138–142.

Lesiński G (2008) Linear landscape elements and bat casualties on roads — an example. *Annales Zoologici Fennici*, 45, 277–280.

Lesiński G, Sikora A, Olszewski A (2010) Bat casualties on a road crossing a mosaic landscape. *European Journal of Wildlife Research*, 57, 217–223.

Medinas D, Marques JT, Mira A (2012) Assessing road effects on bats: the role of landscape, road features, and bat activity on road-kills. *Ecological Research*, 28, 227–237.

Myczko Ł, Sparks TH, Skórka P, Rosin ZM, Kwieciński Z, Górecki MT, Tryjanowski P (2017) Effects of local roads and car traffic on the occurrence pattern and foraging behaviour of bats. *Transportation Research Part D*, 56, 222-228.

O'Donnell CFJ, Christie JE, Simpson W (2006) Habitat use and nocturnal activity of lesser short-tailed bats (Mystacina tuberculata) in comparison with long-tailed bats (Chalinolobus tuberculatus) in temperate rainforest, *New Zealand Journal of Zoology*, 33, 113-124.

Pauli BP, Zollner PA, Haulton GS (2017) Nocturnal habitat selection of bats using occupancy models. *The Journal of Wildlife Management*, 81, 878–891.

Pourshoushtari RD, Pauli BP, Zollner PA, Haulton GS (2018) Road and habitat interact to influence selection and avoidance behavior of bats in Indiana. *Northeastern Naturalist*, 25, 236-247.

Rackow W, Harz O, Schlegel D (1994) Fledermäuse (Chiroptera) als Verkehrsopfer in Niedersachsen. *Nyctalus*, 5, 11–18.

Russell A, Butchkoski C, Saidak L, McCracken, G. (2009) Road-killed bats, highway design, and the commuting ecology of bats. *Endangered Species Research*, 8, 49–60.

Rydell J (1992) Exploitation of Insects around Streetlamps by Bats in Sweden. *Functional Ecology*, 6, 744-750.

Schaub A, Ostwald J, Siemers BM (2008) Foraging bats avoid noise. *The Journal of Experimental Biology*, 211, 3174–3180.

Secco H, Gomes LA, Lemos H, Mayer F, Machado T, Guerreiro M, Gregorin R (2017) Road and landscape features that affect bat roadkills in Southeastern Brazil. *Oecologia Australis,* 21, 323-336.

Siemers BM, Schaub A (2011) Hunting at the highway: traffic noise reduces foraging efficiency in acoustic predators. *Proceedings. Biological Sciences / The Royal Society*, 278, 1646–1652.

Zurcher AA, Sparks DW, Bennett VJ (2010) Why the Bat Did Not Cross the Road? *Acta Chiropterologica*, 12, 337–340.

Appendix 2.1 The behavioural traits of each study species associated with behavioural response to roads. Low-flying, clutter adapted species and species that are more sensitive to artificial lighting are more likely to avoid roads. Low-flying species= <10m, high-flying species >10m.

Species	**Flight height**	**Clutter/edge /open**	**Light sensitivity**
Myotis alcathoe	Low	Clutter adapted	Avoid
Myotis brandti	Low	Clutter adapted	Avoid
Myotis daubentonii	Low	Clutter adapted	Avoid
Myotis mystacinus	Low	Clutter adapted	Avoid
Myotis nattereri	Low	Clutter adapted	Avoid
Plecotus auritus	Low	Clutter adapted	Avoid
Plecotus austriacus	Low	Clutter adapted	Avoid
Rhinolophus hipposideros	Low	Clutter adapted	Avoid
Barbastellus barbastella	Low	Clutter adapted	Do not avoid
Rhinolophus ferrumequinum	Low	Open adapted	Avoid
Pipistrellus nathusii	Low	Edge adapted	Do not avoid
Pipistrellus pipistrellus	Low	Edge adapted	Do not avoid
Pipistrellus pygmaeus	Low	Edge adapted	Do not avoid
Eptesicus serotinus	High	Open adapted	Do not avoid
Nyctalus leisleri	High	Open adapted	Do not avoid
Nyctalus noctula	High	Open adapted	Do not avoid

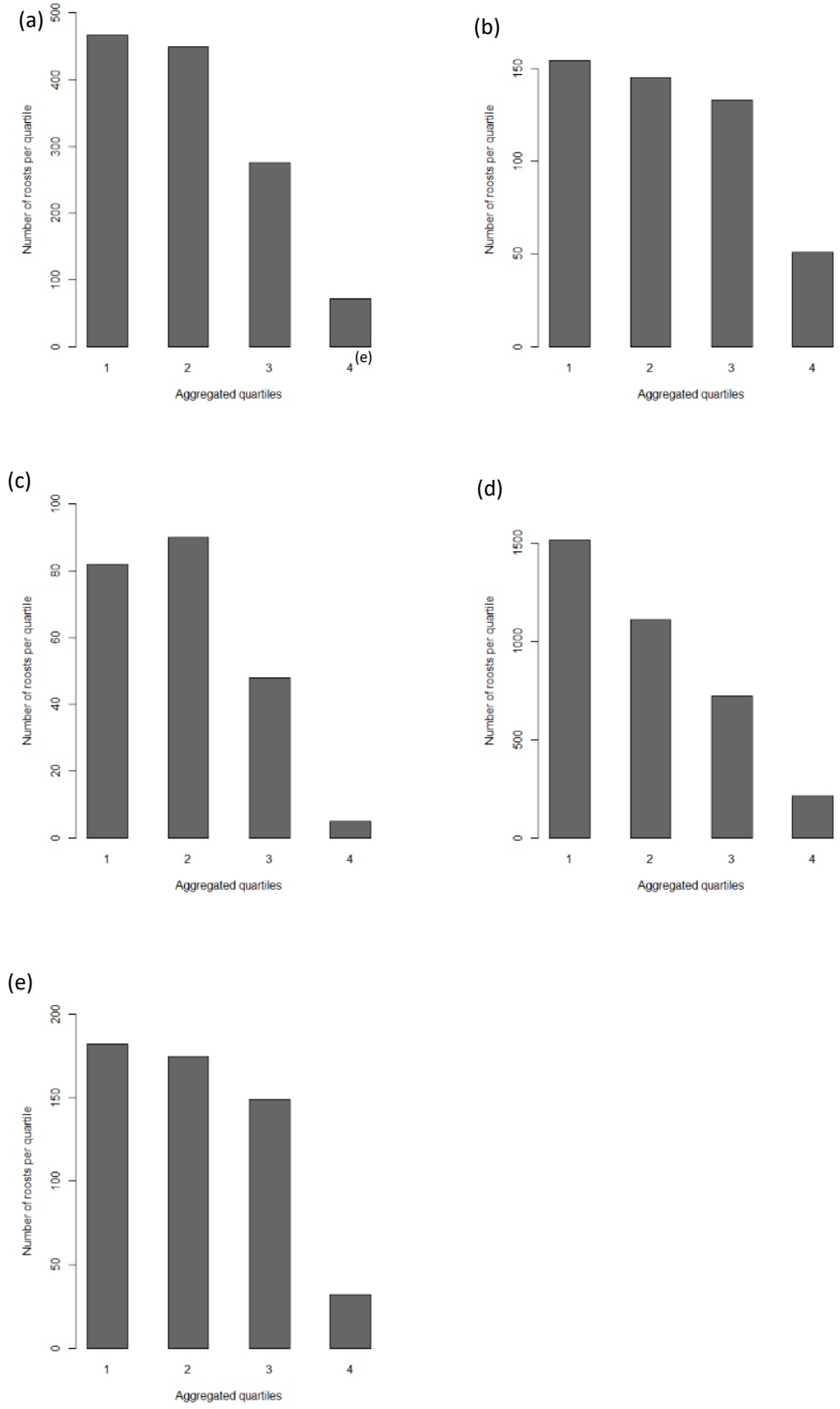
Appendix 3.1 Roosts obtained from the National Bat Monitoring Programme and Natural England datasets by species and for each country.

Species	Number of roosts	England	Scotland	Wales
Pipistrellus pipistrellus	2018	1911	67	40
Plecotus auritus	1261	1209	36	16
Pipistrellus pygmaeus	1025	888	94	43
Pipistrellus sp.	517	416	67	34
Rhinolophus hipposideros	483	312		171
Myotis nattereri	267	255	7	5
Eptesicus serotinus	225	224		1
Myotis mystacinus	142	139	1	2
Rhinolophus ferrumequinum	76	71		5
Myotis brandti	67	66		1
Myotis daubentonii	57	56	1	
Nyctalus noctula	24	24		
Barbastella barbastellus	17	17		
Unidentified species	7	6		1
Pipistrellus nathusii	4	4		
Nyctalus leisleri	3	3		
Myotis mystacinus/Myotis brandti/Myotis alcathoe	3	2		1
Myotis alcathoe	2	2		
Plecotus austriacus	1	1		
Total	**6199**	**5606**	**273**	**320**

Appendix 3.2 The number of occupied and unoccupied patches by country

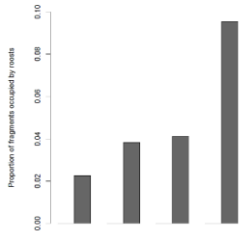
Country	Occupied patches	Unoccupied patches	Total	Percent of occupied patches
England	1545	9606	11151	13.85
Scotland	156	1678	1834	8.50
Wales	138	780	918	15.03
Total	**1839**	**12064**	**13903**	**13.22**

Appendix 3.3 The number of roosts per quartile for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e).

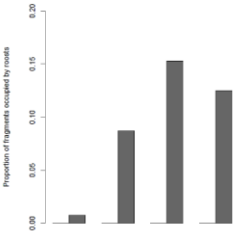


Appendix 3.4 The proportion of patches occupied by at least one roost in each aggregated quartile for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e).

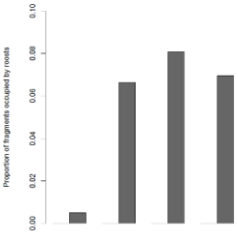
(a)



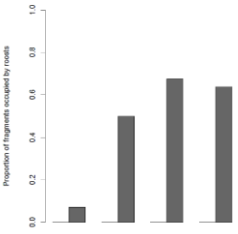
(b)



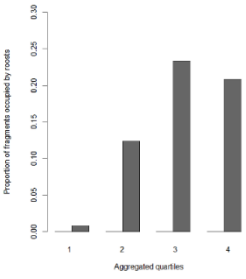
(c)



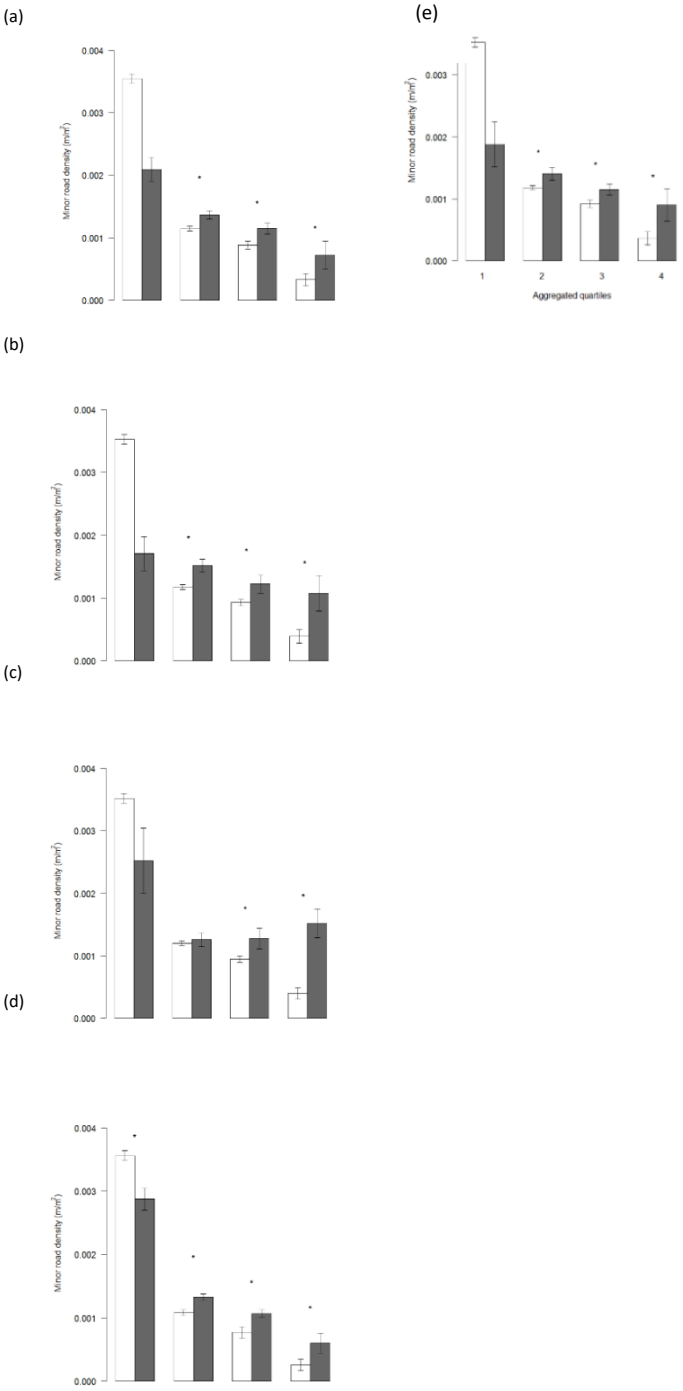
(d)



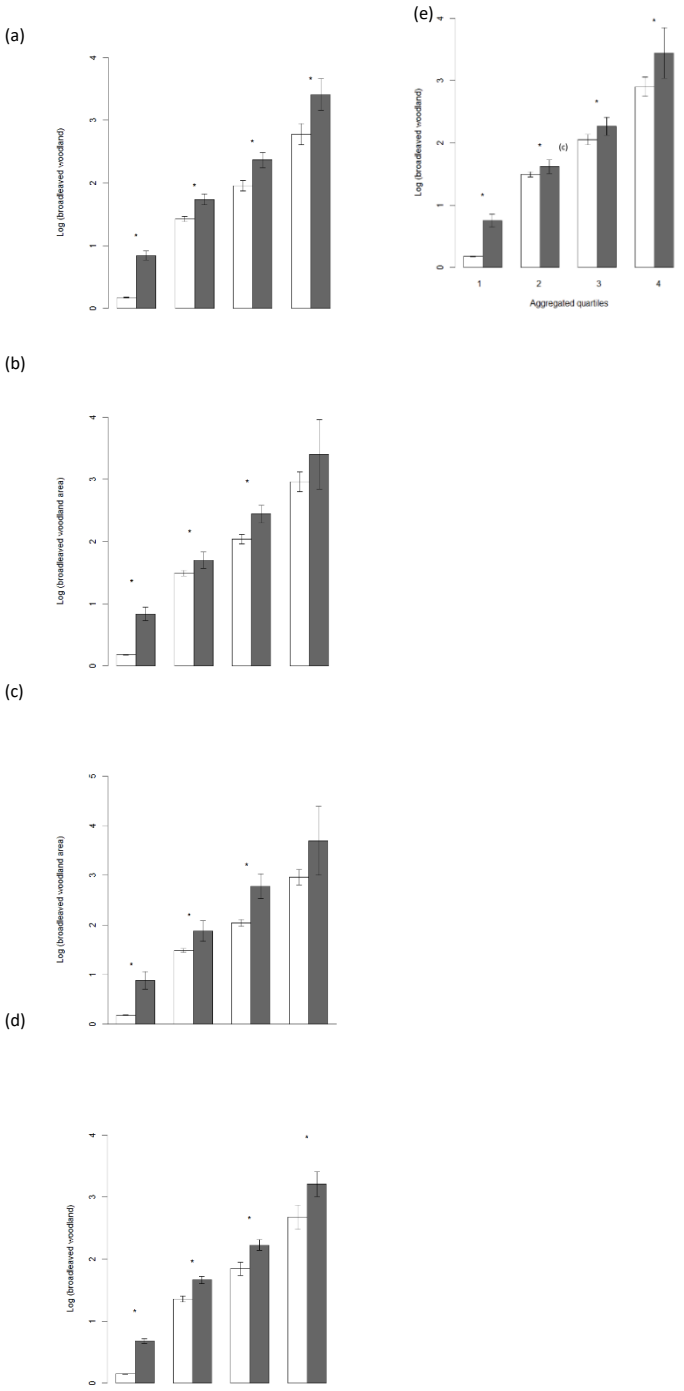
(e)



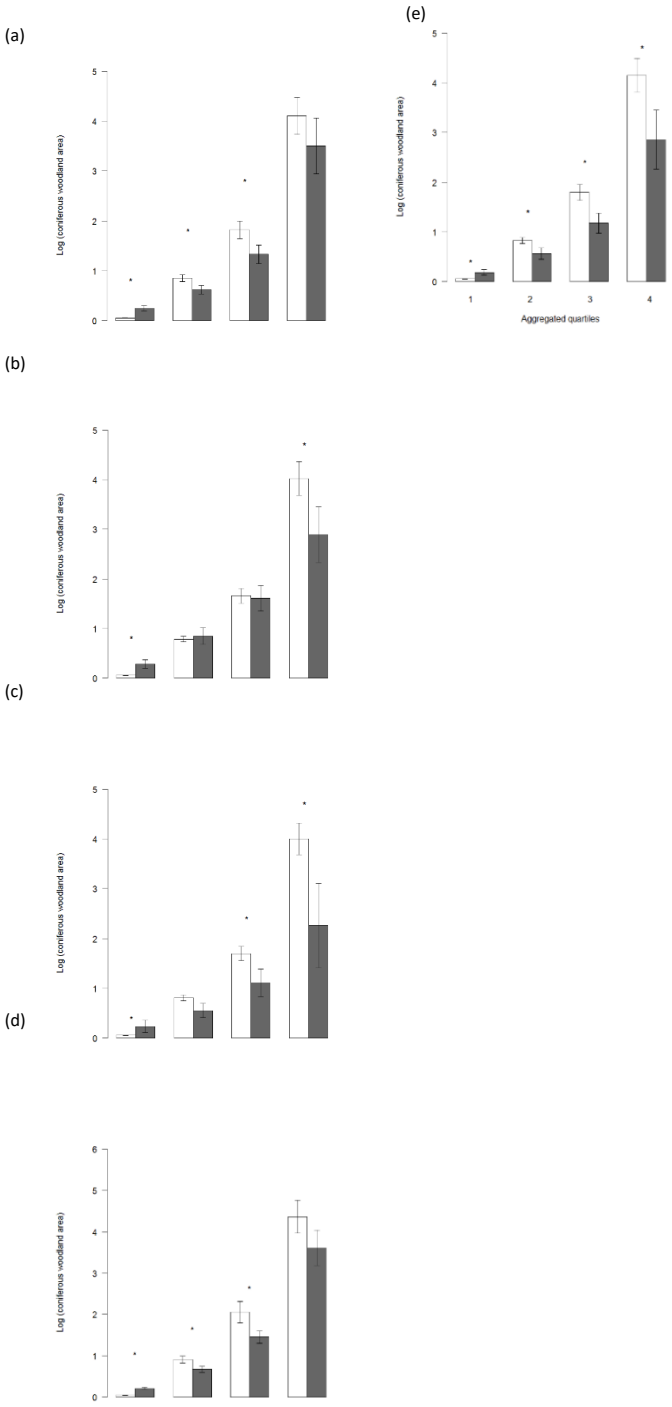
Appendix 3.5 Minor road density (m/m^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e).



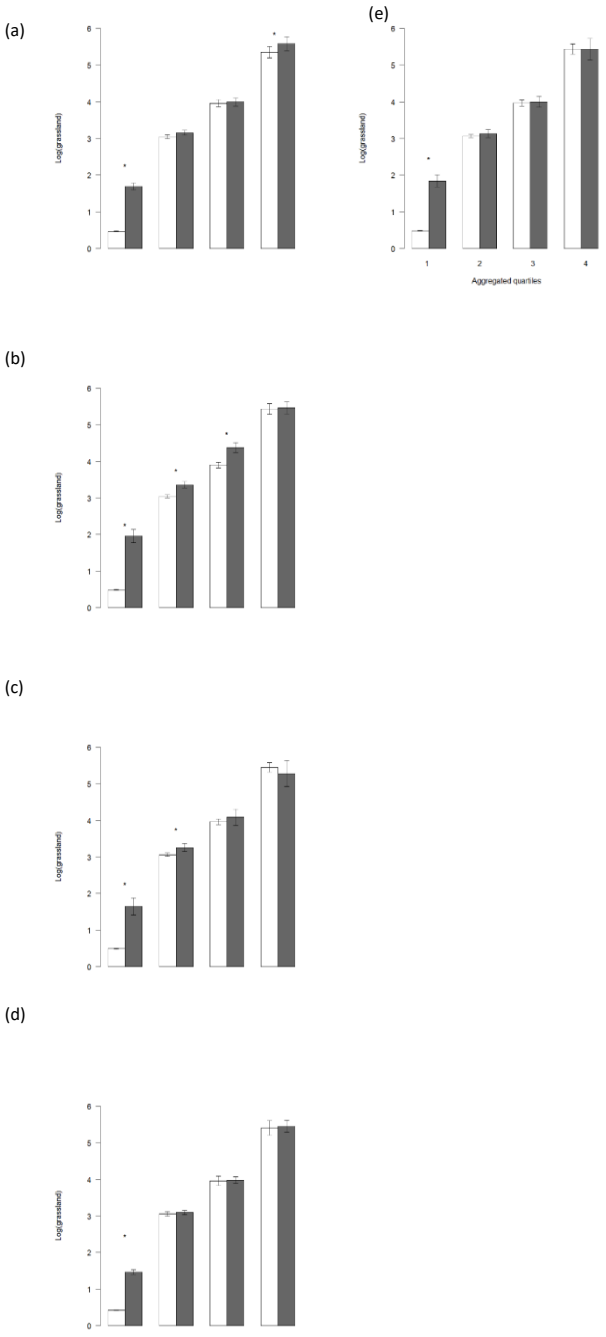
Appendix 3.6 The log of broadleaved woodland area (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e).



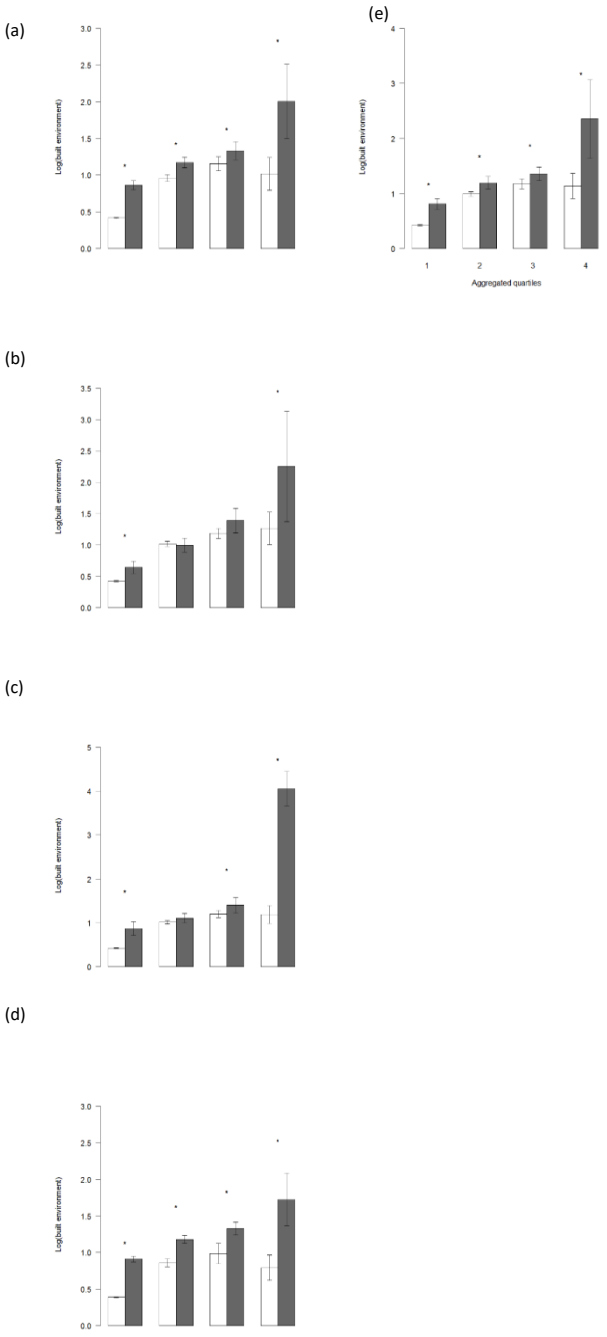
Appendix 3.7 The log of coniferous woodland area (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e).



Appendix 3.8 The log of grassland area (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e).



Appendix 3.9 The log of built environments (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e).



Appendix 3.10 The expected and observed number of roosts in each aggregated quartile. * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

Appendix 2.9		The number of roosts in each quartile		Pearson's chi-squared test		
		Expected	**Observed**	**χ^2**	**Df**	**p-value**
(a)	*Plecotus auritus*					
	Quartile 1	315	466			
	Quartile 2	315	449			
	Quartile 3	315	275	323.21	3	<0.0001****
	Quartile 4	315	71			
(b)	*Rhinolophus hipposideros*					
	Quartile 1	121	154			
	Quartile 2	121	145			
	Quartile 3	121	133	55.55	3	<0.0001****
	Quartile 4	121	51			
(c)	*Eptesicus serotinus*					
	Quartile 1	56	82			
	Quartile 2	56	90			
	Quartile 3	56	48	79.94	3	<0.0001****
	Quartile 4	56	5			
(d)	*Genus Pipistrellus*					
	Quartile 1	891	1516			
	Quartile 2	891	1110			
	Quartile 3	891	723	1036.79	3	<0.0001****
	Quartile 4	891	215			
(e)	*Genus Myotis*					

Quartile 1	135	182			
Quartile 2	135	175	108.64	3	<0.0001****
Quartile 3	135	149			
Quartile 4	135	32			

Appendix 3.11 The expected and observed proportions of occupied patches in each aggregated quartile and the results of Pearson's chi-squared tests for *Plecotus auritus* (a), *Rhinolophus hipposideros* (b), *Eptesicus serotinus* (c), *Genus Pipistrellus* (d) *and Genus Myotis* (e). * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

		Proportion of occupied patches		Pearson's chi-squared test		
		Expected	**Observed**	**χ^2**	**Df**	**p-value**
(a)	*Plecotus auritus*					
	Quartile 1	0.049	0.02			
	Quartile 2	0.049	0.26	1887.6	3	<0.0001****
	Quartile 3	0.049	0.35			
	Quartile 4	0.049	0.37			
(b)	*Rhinolophus hipposideros*					
	Quartile 1	0.016	0.0075			
	Quartile 2	0.016	0.0870	766.83	3	<0.0001****
	Quartile 3	0.016	0.1527			
	Quartile 4	0.016	0.125			
(c)	*Eptesicus serotinus*					
	Quartile 1	0.011	0.0049			
	Quartile 2	0.011	0.0664	462.32	3	<0.0001****
	Quartile 3	0.011	0.0806			
	Quartile 4	0.011	0.0694			

(d)	*Genus Pipistrellus*					
	Quartile 1	0.11	0.0707			
	Quartile 2	0.11	0.4982	2751.4	3	<0.0001****
	Quartile 3	0.11	0.6743			
	Quartile 4	0.11	0.6388			
(e)	*Genus Myotis*					
	Quartile 1	0.022	0.0084			
	Quartile 2	0.022	0.1237	1340.7	3	<0.0001****
	Quartile 3	0.022	0.2334			
	Quartile 4	0.022	0.2083			

Appendix 3.12 Comparisons of occupied and unoccupied patches in each aggregated quartile for minor road density broadleaved woodland, coniferous woodland, grassland and built environments. Results are reported for each species. * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001

		Mean values		Wilcoxon rank sum	
	Minor road density (m/m^2)	**Unoccupied**	**Occupied**	**W**	**p-value**
(a)	*Plecotus auritus*				
	Quartile 1	0.00354737	0.002092163	1948500	0.3747
	Quartile 2	0.001145565	0.001366508	54709	<0.0001****
	Quartile 3	0.0008790631	0.001147098	9447	<0.0001****
	Quartile 4	0.0003281978	0.0007207637	403	<0.05*
(b)	*Rhinolophus hipposideros*				
	Quartile 1	0.003525513	0.001706696	644000	0.1529
	Quartile 2	0.001175188	0.001518098	16777	<0.0001****
	Quartile 3	0.0009282421	0.001223245	5405	<0.001***
	Quartile 4	0.0003898735	0.001074166	69	<0.001***
(c)	*Eptesicus serotinus*				
	Quartile 1	0.0035168	0.002516958	378470	0.5632
	Quartile 2	0.001201009	0.001261689	21110	0.1737
	Quartile 3	0.0009468582	0.001274552	2707	<0.001***
	Quartile 4	0.0003974815	0.001519653	7	<0.001***
(d)	*Genus Pipistrellus*				
	Quartile 1	0.003560306	0.002874321	4819200	<0.001***
	Quartile 2	0.001081836	0.001329094	67734	<0.0001****
	Quartile 3	0.0007734919	0.001069789	8505	<0.0001****
	Quartile 4	0.0002562512	0.0005992824	376	<0.01**
(e)	*Genus Myotis*				
	Quartile 1	0.003525784	0.001879144	720670	0.1589

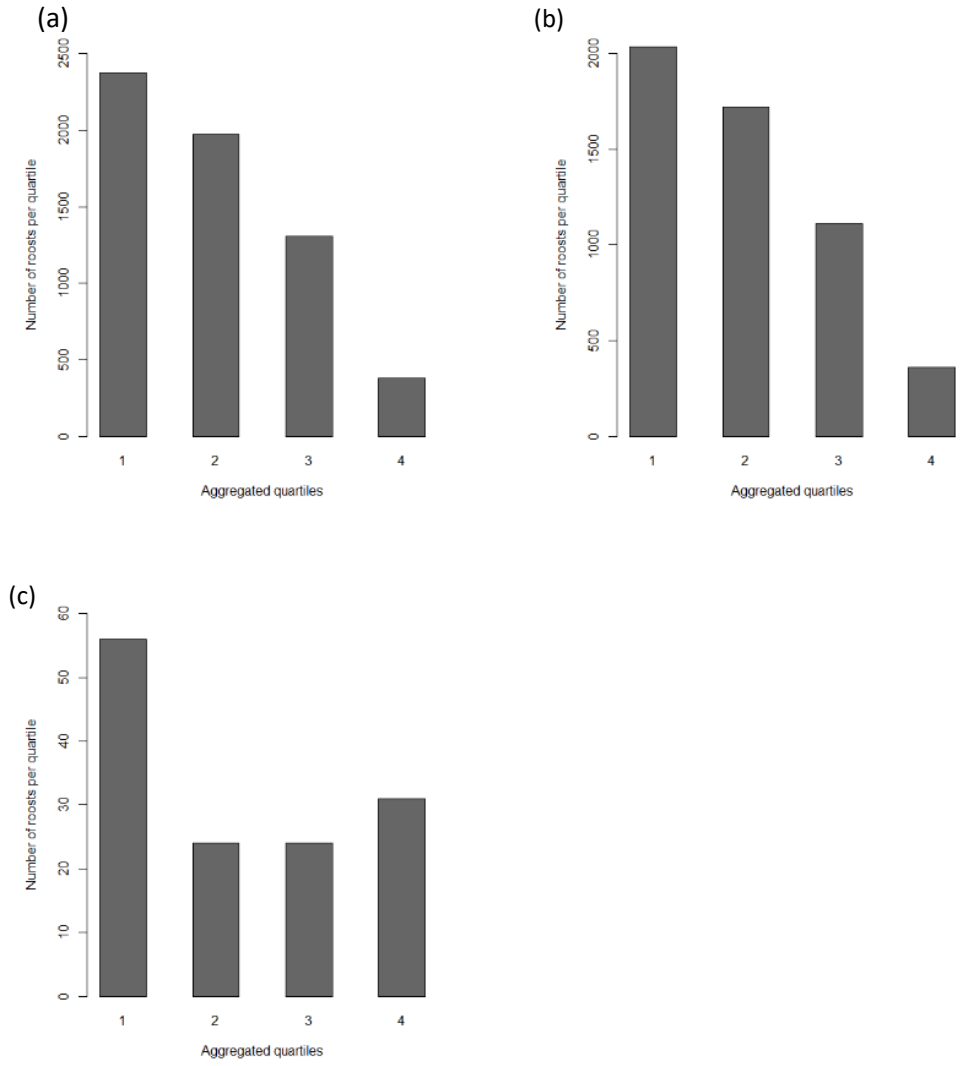
	Quartile 2	0.00117711	0.001402881	30770	<0.0001****
	Quartile 3	0.0009187288	0.00115251	7720	<0.001***
	Quartile 4	0.0003626424	0.0009039271	147	<0.001***
	Broadleaved woodland (km^2)	**Unoccupied**	**Occupied**	**W**	**p-value**
(a)	*Plecotus auritus*				
	Quartile 1	0.28	1.86	475020	<0.0001****
	Quartile 2	3.87	6.04	53788	<0.0001****
	Quartile 3	7.44	12.17	9110	<0.0001****
	Quartile 4	17.45	36.34	289	<0.001***
(b)	*Rhinolophus hipposideros*				
	Quartile 1	0.31	1.63	129920	<0.0001****
	Quartile 2	4.37	5.34	23354	<0.001***
	Quartile 3	8.59	11.97	4793	<0.0001****
	Quartile 4	22.22	40.71	202	0.1678
(c)	*Eptesicus serotinus*				
	Quartile 1	0.31	2.12	108470	<0.0001****
	Quartile 2	4.22	7.65	16330	<0.0001****
	Quartile 3	8.29	18.41	1783	<0.0001****
	Quartile 4	22.57	50.89	82	0.05971
(d)	*Genus Pipistrellus*				
	Quartile 1	0.24	1.38	1670300	<0.0001****
	Quartile 2	3.48	5.43	67290	<0.0001****
	Quartile 3	6.32	10.45	8676	<0.0001****
	Quartile 4	15.41	29.70	309	<0.001***
(e)	*Genus Myotis*				
	Quartile 1	0.31	1.45	186990	<0.0001****
	Quartile 2	4.38	4.94	36028	<0.05*
	Quartile 3	8.60	10.77	8766	<0.05*
	Quartile 4	20.36	40.42	264	<0.05*

	Coniferous woodland (km^2)	Unoccupied	Occupied	W	p-value
(a)	*Plecotus auritus*				
	Quartile 1	0.10	0.48	820200	<0.0001****
	Quartile 2	2.78	1.41	83176	<0.05*
	Quartile 3	13.32	6.32	16416	<0.01**
	Quartile 4	103.96	73.55	740	0.1253
(b)	*Rhinolophus hipposideros*				
	Quartile 1	0.10	0.50	201350	<0.0001****
	Quartile 2	2.43	2.20	26807	0.09769
	Quartile 3	11.54	7.07	7515	0.6819
	Quartile 4	102.35	23.99	436	<0.01**
(c)	*Eptesicus serotinus*				
	Quartile 1	0.10	0.61	184830	<0.0001****
	Quartile 2	2.51	1.07	26379	0.1392
	Quartile 3	11.56	2.88	5498	<0.05*
	Quartile 4	98.56	12.10	285	<0.01**
(d)	*Genus Pipistrellus*				
	Quartile 1	0.08	0.41	2587100	<0.0001****
	Quartile 2	3.04	1.78	107520	<0.01**
	Quartile 3	16.25	8.26	16500	<0.001***
	Quartile 4	117.63	78.38	750	0.0756
(e)	*Genus Myotis*				
	Quartile 1	0.11	0.26	306910	<0.0001****
	Quartile 2	2.58	1.20	46346	<0.05*
	Quartile 3	12.74	4.70	13602	<0.001***
	Quartile 4	109.12	29.62	670	<0.001***
	Grassland (km^2)	**Unoccupied**	**Occupied**	**W**	**p-value**
(a)	*Plecotus auritus*				
	Quartile 1	1.40	6.40	500400	<0.0001****

	Quartile 2	24.20	25.53	69550	0.1013
	Quartile 3	64.52	63.54	13941	0.8091
	Quartile 4	242.40	299.31	430	<0.05*
(b)	*Rhinolophus hipposideros*				
	Quartile 1	1.47	8.65	124610	<0.0001****
	Quartile 2	24.03	30.13	21219	<0.0001****
	Quartile 3	59.81	88.37	4767	<0.0001****
	Quartile 4	266.88	241.79	257	0.658
(c)	*Eptesicus serotinus*				
	Quartile 1	1.50	6.36	123540	<0.0001****
	Quartile 2	24.36	27.34	19868	<0.05*
	Quartile 3	63.73	69.24	4220	0.6295
	Quartile 4	267.82	209.15	200	0.4784
(d)	*Genus Pipistrellus*				
	Quartile 1	1.23	5.40	1665600	<0.0001****
	Quartile 2	24.67	24.45	94348	0.8057
	Quartile 3	62.86	64.81	13089	0.8806
	Quartile 4	253.02	269.80	564	0.6964
(e)	*Genus Myotis*				
	Quartile 1	1.47	7.58	165610	<0.0001****
	Quartile 2	24.46	25.25	39332	0.4202
	Quartile 3	63.84	65.27	10708	0.935
	Quartile 4	264.65	260.28	399	0.6978
	Built environment (km^2)	**Unoccupied**	**Occupied**	**W**	**p-value**
(a)	*Plecotus auritus*				
	Quartile 1	0.76	1.76	919300	<0.0001****
	Quartile 2	2.21	2.87	58110	<0.0001****
	Quartile 3	3.31	3.86	11384	<0.01**
	Quartile 4	3.47	17.22	315	<0.001***

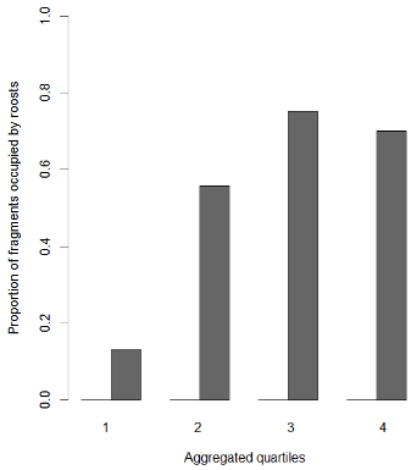
(b)	*Rhinolophus hipposideros*				
	Quartile 1	0.78	1.17	387760	<0.0001****
	Quartile 2	2.42	2.06	30133	0.9421
	Quartile 3	3.34	4.43	6552	0.06542
	Quartile 4	7.33	17.67	149	<0.05*
(c)	*Eptesicus serotinus*				
	Quartile 1	0.78	1.93	201720	<0.0001****
	Quartile 2	2.39	2.28	20081	0.05548
	Quartile 3	3.50	3.58	3424	<0.05*
	Quartile 4	4.68	61.43	6	<0.001***
(d)	*Genus Pipistrellus*				
	Quartile 1	0.70	1.94	2236000	<0.0001****
	Quartile 2	1.90	2.87	63219	<0.0001****
	Quartile 3	2.82	3.83	8980	<0.0001****
	Quartile 4	1.41	12.70	276	<0.001***
(e)	*Genus Myotis*				
	Quartile 1	0.78	1.61	352400	<0.0001****
	Quartile 2	2.29	3.06	33250	<0.01**
	Quartile 3	3.48	3.58	8587	<0.01**
	Quartile 4	4.84	22.99	187	<0.001***

Appendix 3.13 The number of roosts per quartile with 0-80% built environments (a), 0-20% built environments (b) and 80-100% built environments (c).

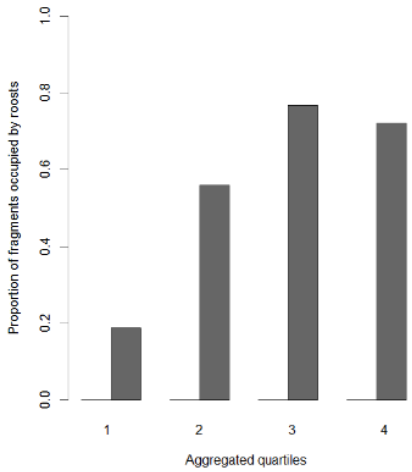


Appendix 3.14 The proportion of patches occupied by at least one roost in each aggregated quartile for patches with 0-80% built environments (a), 0-20% built environments (b), and 80-100% (c).

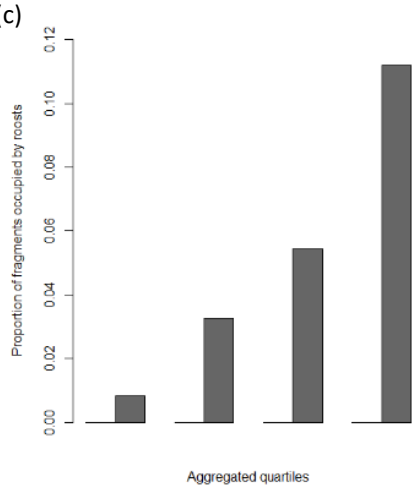
(a)



(b)

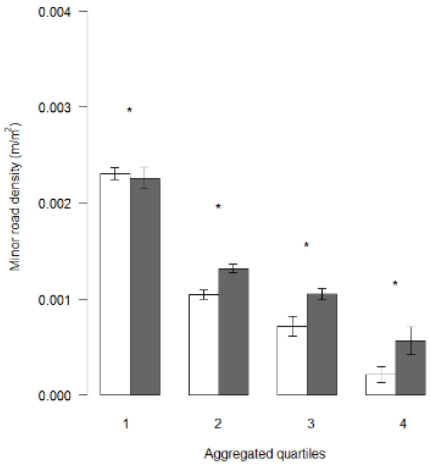


(c)

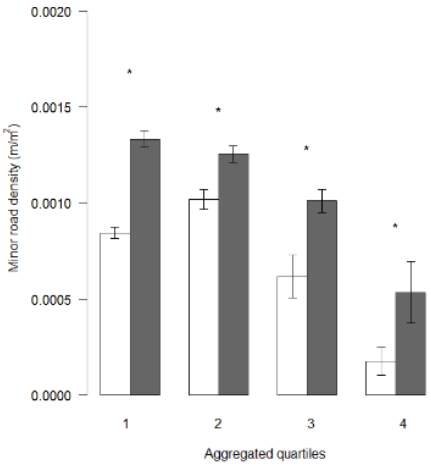


Appendix 3.15 Minor road density (m/m^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for patches with 0-80% built environments (a), 0-20% built environments (b), and 80-100% built environments (c).

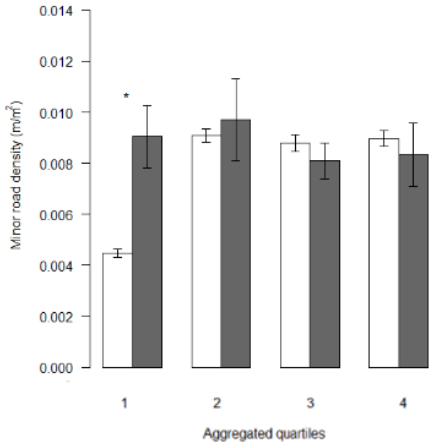
(a)



(b)

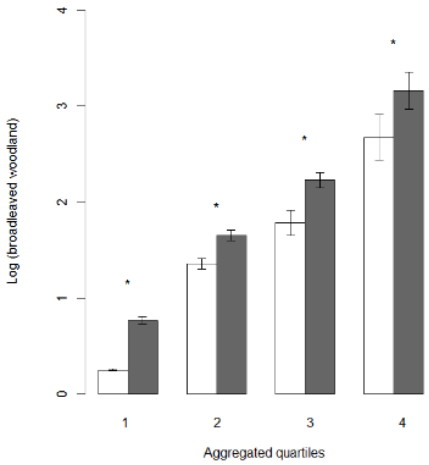


(c)

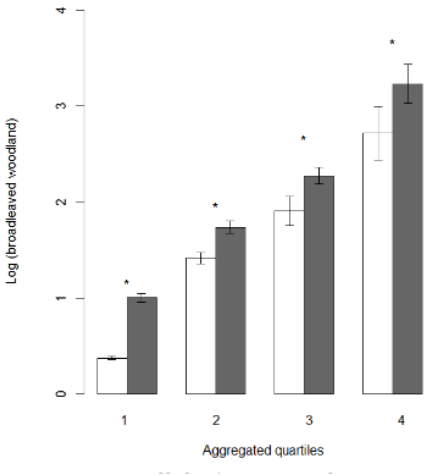


Appendix 3.16 The log of broadleaved woodland area (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for patches with 0-80% built environments (a), 0-20% built environments (b) and 80-100% built environments (c).

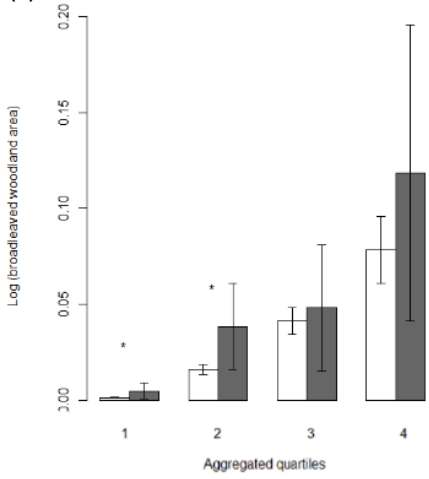
(a)



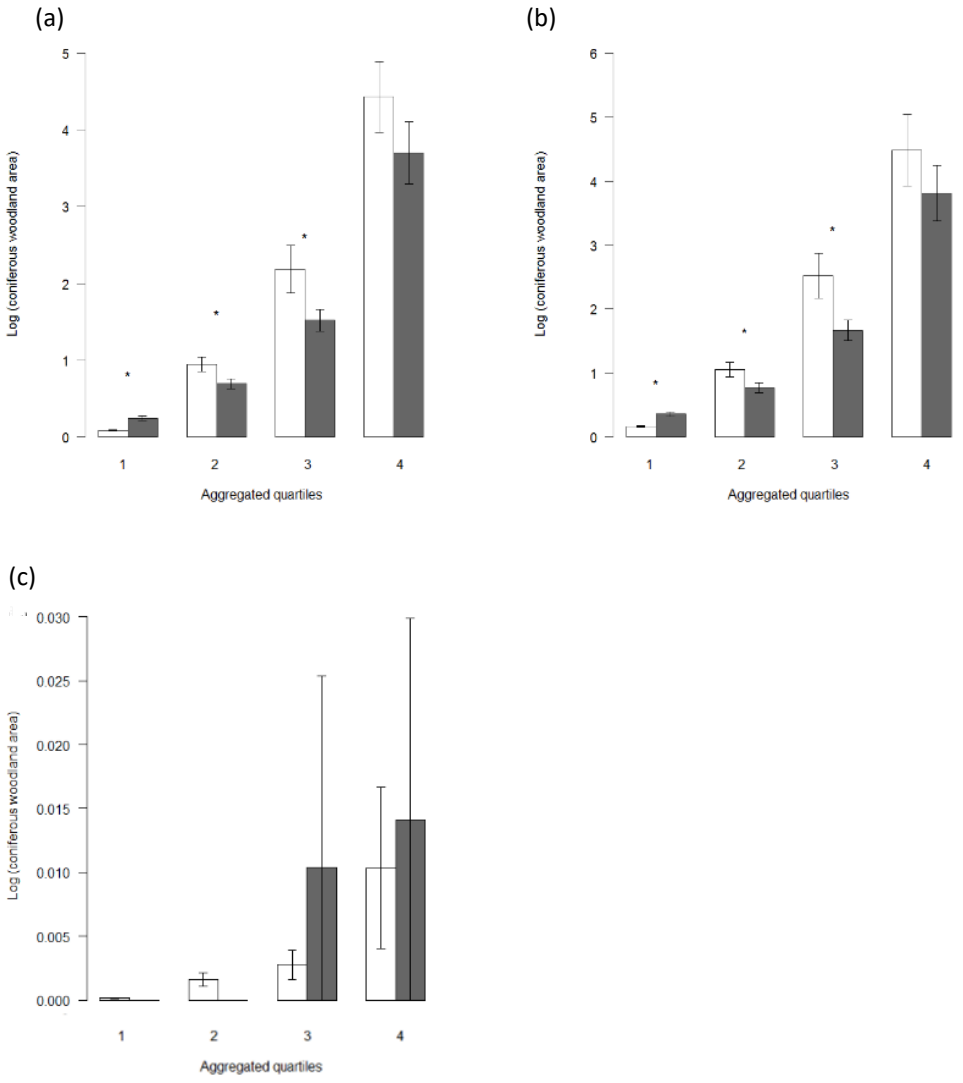
(b)



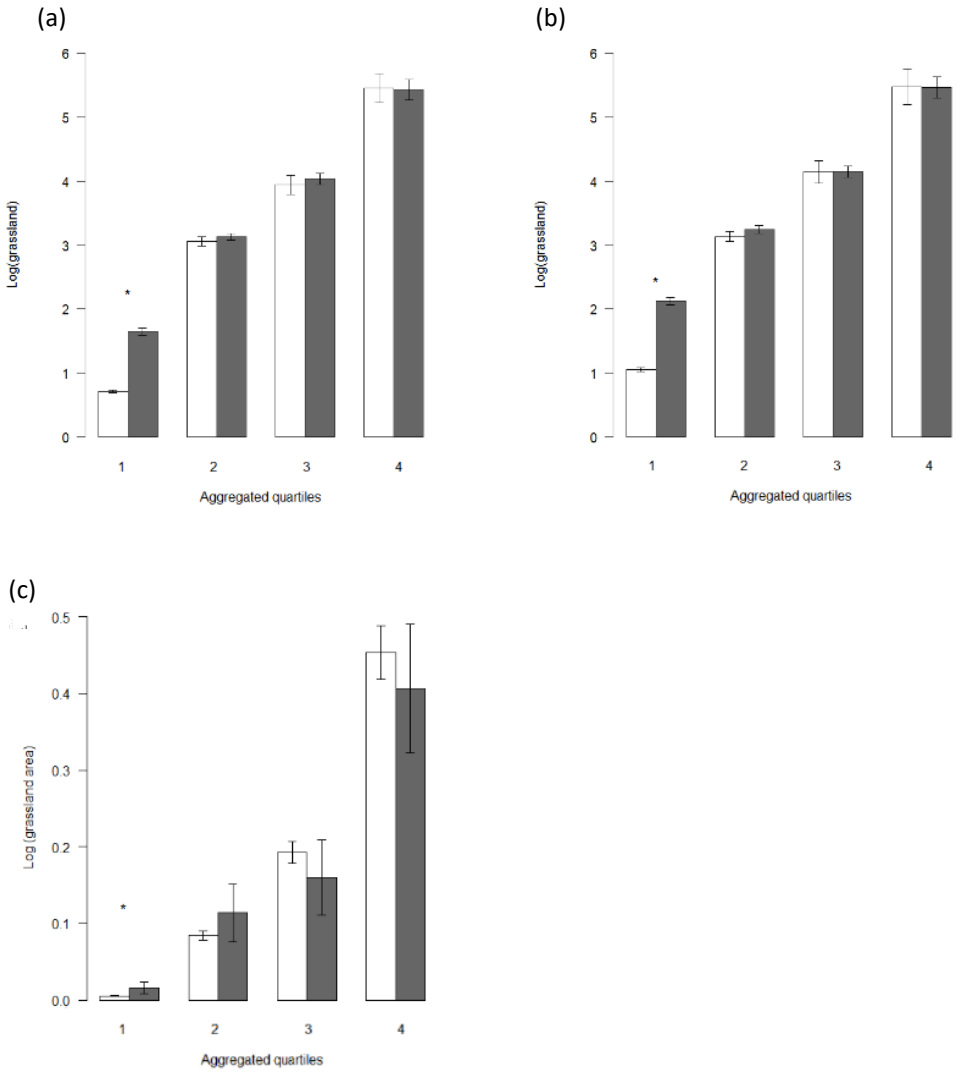
(c)



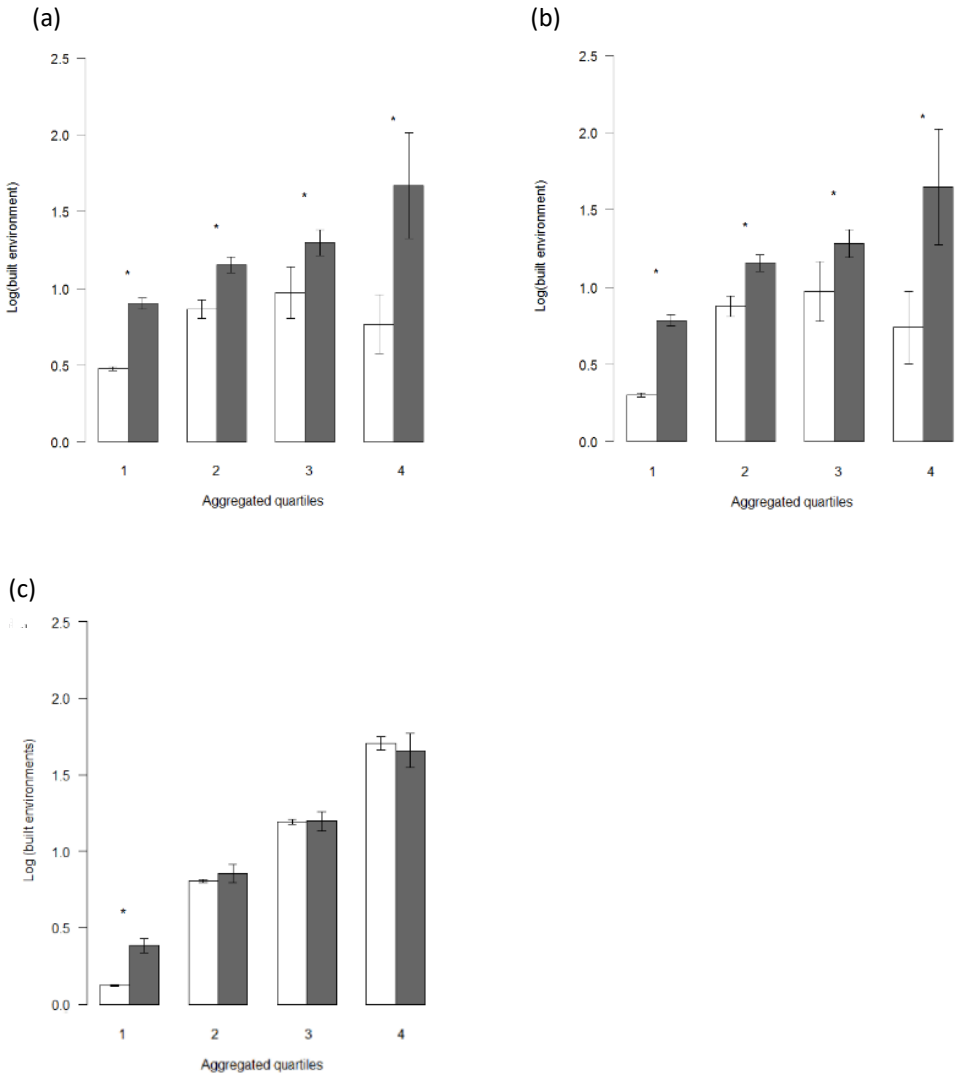
Appendix 3.17 The log of coniferous woodland area (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for patches with 0-80% built environments (a), 0-20% built environments (b) and 80-100% built environments (c).



Appendix 3.18 The log grassland area (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for patches with 0-80% built environments (a), 0-20% built environments (b) and 80-100% built environments (c).



Appendix 3.19 The log of built environments (km^2) in occupied (grey columns) and unoccupied (white columns) patches in each aggregated quartile for patches with 0-80% built environments (a), 0-20% built environments (b) and 80-100% built environments (c).



Appendix 3.20 The expected and observed number of roosts for each aggregated quartile and the results of Pearson's chi-squared tests for patches with 0-80%, 0-20% and 80-100% built environments. * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

		Number of roosts in each quartile		Pearson's chi-squared test		
		Expected	**Observed**	**χ^2**	**df**	**p-value**
(a)	*0-80%*					
	Quartile 1	1509	2377			
	Quartile 2	1509	1974			
	Quartile 3	1509	1308	1516.78	3	<0.0001****
	Quartile 4	1509	378			
(b)	*0-20%*					
	Quartile 1	1308	2036			
	Quartile 2	1308	1723			
	Quartile 3	1308	1113	1253.01	3	<0.0001****
	Quartile 4	1308	360			
(c)	80-100%					
	Quartile 1	135	56			
	Quartile 2	135	24			
	Quartile 3	135	24	308.88	3	<0.0001****
	Quartile 4	135	31			

Appendix 3.21 The expected and observed proportions of occupied patches in each aggregated quartile and the results of Pearson's chi-squared tests for patches with 0-80% built environments (a) and 0-20% built environments (b). * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

			Proportion of occupied patches		Pearson's chi-squared test		
			Expected	**Observed**	**χ^2**	**df**	**p-value**
(a)	*0-80%*						
		Quartile 1	0.20	0.13			
		Quartile 2	0.20	0.55			
		Quartile 3	0.20	0.75	1625.3	3	<0.0001****
		Quartile 4	0.20	0.7			
(b)	*0-20%*						
		Quartile 1	0.27	0.18			
		Quartile 2	0.27	0.55			
		Quartile 3	0.27	0.76	841.14	3	<0.0001****
		Quartile 4	0.27	0.72			
(c)	*80-100%*						
		Quartile 1	0.012	0.008			
		Quartile 2	0.012	0.016	127.76	3	<0.0001****
		Quartile 3	0.012	0.018			
		Quartile 4	0.012	0.028			

Appendix 3.22 The mean values of minor road density, broadleaved woodland, coniferous woodland, grassland and built environments in occupied and unoccupied patches with 0-80%, 0-20% and 80-100% built environments. * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

			Mean values		Wilcoxon rank sum	
	Minor road density (m/m^2)		**Unoccupied**	**Occupied**	**W**	**p-value**
(a)	0-80%					
		Quartile 1	0.0023	0.0022	2833100	<0.0001****
		Quartile 2	0.0010	0.0013	58580	<0.0001****
		Quartile 3	0.0007	0.0010	6410	<0.0001****
		Quartile 4	0.0002	0.0005	295	<0.01**
(b)	0-20%					
		Quartile 1	0.00084	0.00133	744270	<0.0001****
		Quartile 2	0.00101	0.00125	40575	<0.0001****
		Quartile 3	0.00061	0.00101	3699	<0.0001****
		Quartile 4	0.00017	0.00053	208	<0.01**
(c)	80-100%					
		Quartile 1	0.00445	0.00904	34109	<0.0001****
		Quartile 2	0.00908	0.00970	3467	0.55
		Quartile 3	0.00879	0.00808	1933	0.39
		Quartile 4	0.00896	0.00832	992	0.09
	Broadleaved woodland (km^2)		**Unoccupied**	**Occupied**	**W**	**p-value**
(a)	0-80%					
		Quartile 1	0.41	1.59	1233900	<0.0001****
		Quartile 2	3.48	5.33	61286	<0.0001****
		Quartile 3	6.00	10.39	6350	<0.0001****
		Quartile 4	15.68	28.45	294	<0.01**
(b)	0-20%					

	Quartile 1	0.67	2.30	474140	<0.0001****
	Quartile 2	3.75	5.97	41434	<0.0001****
	Quartile 3	6.94	10.83	4851	<0.0001****
	Quartile 4	16.67	30.34	206	<0.01**
(c)	80-100%				
	Quartile 1	0.0013	0.0048	59602	<0.001***
	Quartile 2	0.0166	0.0401	2093.5	<0.01**
	Quartile 3	0.0435	0.0511	1722	0.9
	Quartile 4	0.0861	0.1375	661	0.3
	Coniferous woodland (km^2)	**Unoccupied**	**Occupied**	**W**	**p-value**
(a)	0-80%				
	Quartile 1	0.15	0.49	1841200	<0.0001****
	Quartile 2	3.29	1.83	97249	<0.01**
	Quartile 3	18.84	8.68	13201	<0.001***
	Quartile 4	126.97	81.11	636	0.1216
(b)	0-20%				
	Quartile 1	0.32	0.73	720280	<0.0001****
	Quartile 2	4.02	2.10	68096	<0.001***
	Quartile 3	25.62	10.08	9675	<0.0001****
	Quartile 4	138.95	88.18	451	0.2208
(c)	80-100%				
	Quartile 1	0.00013	0	76002	0.37
	Quartile 2	0.00162	0	4240	0.15
	Quartile 3	0.00282	0.01	1485	0.22
	Quartile 4	0.01098	0.01	605.5	0.09
	Grassland (km^2)	**Unoccupied**	**Occupied**	**W**	**p-value**
(a)	0-80%				
	Quartile 1	2.07	6.37	1301000	<0.0001****
	Quartile 2	24.76	25.43	83125	0.29

		Quartile 3	62.18	68.20	9706	0.3261
		Quartile 4	266.67	263.33	520	0.9493
(b)	0-20%					
		Quartile 1	3.83	9.99	518530	<0.0001****
		Quartile 2	27.24	28.71	55331	0.1241
		Quartile 3	78.00	75.79	7180	0.9816
		Quartile 4	279.40	273.26	378	0.9555
(c)	80-100%					
		Quartile 1	0.005	0.016	44376	<0.0001****
		Quartile 2	0.090	0.123	2857.5	0.09
		Quartile 3	0.219	0.178	1979	0.3
		Quartile 4	0.602	0.519	917	0.2
	Built environment (km^2)		**Unoccupied**	**Occupied**	**W**	**p-value**
(a)	0-80%					
		Quartile 1	0.87	1.92	1720300	<0.0001****
		Quartile 2	1.95	2.78	59823	<0.0001****
		Quartile 3	2.77	3.78	7289	<0.0001****
		Quartile 4	1.36	12.01	246	<0.001***
(b)	0-20%					
		Quartile 1	0.47	1.51	502640	<0.0001****
		Quartile 2	1.91	2.70	40978	<0.0001****
		Quartile 3	2.82	3.55	5086	<0.001***
		Quartile 4	1.33	12.48	188	<0.01**
(c)	80-100%					
		Quartile 1	0.14	0.48	18580	<0.0001****
		Quartile 2	1.24	1.36	2848	0.08
		Quartile 3	2.32	2.32	1683	0.94
		Quartile 4	4.67	4.36	890	0.37

Appendix 4.1 Systematic review of literature pertaining to endocrinological and other stress related responses to conservation relevant variables

A total of 200 studies were assessed for relevance in two databases (Google Scholar and Web of Science) using the terms “animals AND stress AND conservation OR conservation physiology”. The final dataset includes 71 relevant studies.

References for Figure 4.6

Allen LC, Turmelle AS, Widmaier EP, Hristov NI, McCracken GF, Kunz TH (2010) Variation in Physiological Stress between Bridge- and Cave-Roosting Brazilian Free-Tailed Bats. *Conservation Biology*, 25, 374–381

Alzaid A, Hori TS, Hall JR, Rise ML, Gamperl AK (2015) Cold-induced changes in stress hormone and steroidogenic transcript levels in cunner (*Tautogolabrus adspersus*), a fish capable of metabolic depression. *General and Comparative Endocrinology*, 224, 126–135

Bauer CM, Staff NK, Bernard AB, Trevino JM, Ho JM, Romero LM, Ebensperger LA, Hayes LD (2013) Habitat type influences endocrine stress response in the degu (*Octodon degus*). *General and Comparative Endocrinology*, 186, 136-144

Baxter-Gilbert JH, Riley JL, Mastromnaco GF, Litzgus JD, Lesbarrères D (2014) A novel technique to measure chronic levels of corticosterone in turtles living around a major roadway. *Conservation Physiology*, 2, doi:10.1093/conphys/cou036.

Blas J, Bortolotti GR, Tella JL, Baos R, Merchant TA (2007) Stress response during development predicts fitness in a wild, long lived vertebrate. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 8880-8884

Bonier F, Moore IT, Martin PR, Robertson RJ (2009) The relationship between fitness and baseline glucocorticoids in a passerine bird. *General and Comparative Endocrinology*, 163, 208–213

Brearley G, McAlpine C, Bell S, Bradley A (2012) Influence of urban edges on stress in an arboreal mammal: a case study of squirrel gliders in southeast Queensland, Australia. *Landscape Ecology*, 27, 1407–1419

Cabezas S, Blas J, Marchant TA, Moreno S (2007) Physiological stress levels predict survival probabilities in wild rabbits. *Hormones and Behavior*, 51, 313-320

Cantarelli VI, Perez-Rueda M, Kowalewski MM, Mastromonaco GF, Ponzio MF (2017) Validation of an enzyme immunoassay and comparison of fecal cortisol metabolite levels in black and gold howler monkeys (*Alouatta caraya*) inhabiting fragmented and continuous areas of the humid Chaco region, Argentina. *American Journal of Primatology*, 79, e22625

Chapman CA, Wasserman MD, Gillespie TR, Speirs ML, Lawes MJ, Saj TL, Ziegler TE (2006) Do food availability, parasitism, and stress have synergistic effects on Red Colobus populations living in forest fragments? *American Journal of Physical Anthropology*, 131, 525-534

Chávez-Zichinelli CA, MacGregor-Fors I, Quésada J, Talamás Rohana P, Romano MC, Valdéz R, Schondube JE (2013) How stressed are birds in an urbanizing landscape? Relationships between the physiology of birds and three levels of habitat alteration. *The Condor*, 115, 84-92

Creel S, Fox JE, Hardy A, Sands J, Garrott B, Peterson RO (2002) Snowmobile activity and glucocorticoid stress responses in wolves and elk. *Conservation Biology*, 16, 809-814

Crino OL, Klaasen Van Ooschot B, Johnson EE, Malisch JL, Breuener CW (2011) Proximity to a high traffic road: Glucocorticoid and life history consequences for nestling white-crowned sparrows. *General and Comparative Endocrinology*, 173, 323–332

Crino OL, Johnson EE, Blickley JL, Patricelli GL, Breuner CW (2013) Effects of experimentally elevated traffic noise on nestling white-crowned sparrow stress physiology, immune function and life history. *The Journal of Experimental Biology*, 216, 2055-2062

Cyr NE, Romero LM (2008) Fecal glucocorticoid metabolites of experimentally stressed captive and free-living starlings: Implications for conservation research. *General and Comparative Endocrinology*, 158, 20–28

Dantzer B, McAdam AG, Palme R, Fletcher QE, Boutin S, Humphries MM, Boonstra R (2010) Fecal cortisol metabolite levels in free-ranging North American red squirrels: Assay validation and the effects of reproductive condition. *General and Comparative Endocrinology*, 167, 279-286

Dantzer B, McAdam AG, Palme R, Boutin S, Boonstra R (2011) How does diet affect fecal steroid hormone metabolite concentrations? An experimental examination in red squirrels. *General and Comparative Endocrinology*, 174, 124-131

Dantzer B, Santicchia F, van Kesteren F, Palme R, Martinoli A, Wauters LA (2016) Measurement of fecal glucocorticoid metabolite levels in Eurasian red squirrels (*Sciurus vulgaris*): effects of captivity, sex, reproductive condition, and season. *Journal of Mammalogy*, 97, 1385-1398

Delehanty B, Boonstra R (2009) Impact of live trapping on stress profiles of Richardson's ground squirrel (*Spermophilus richardsonii*). *General and Comparative Endocrinology*, 160, 176-182

Dickens MJ, Earle KA, Romero LM (2009) Initial transference of wild birds to captivity alters stress physiology. *General and Comparative Endocrinology*, 160, 76–83

Ditmer MA, Garshelis DL, Noyce KV, Laske TG, Iaizzo PA, Burk TE, Forester JD, Fieberg JR (2015) Behavioral and physiological responses of American black bears to landscape features within an agricultural region. *Ecosphere*, 6, (art 28)

Eikenaar C, Müller F, Klinner T, Bairlein F (2015) Baseline corticosterone levels are higher in migrating than sedentary common blackbirds in autumn, but not in spring. *General and Comparative Endocrinology*, 224, 121–125

Ellenberg U, Mattern T, Seddon PJ, Luna Jorquera GJ (2006) Physiological and reproductive consequences of human disturbance in Humboldt penguins: The need for species-specific visitor management. *Biological Conservation*, 133, 95-106

Ellenberg U, Setiawan AN, Cree A, Houston DM, Seddon PJ (2007) Elevated hormonal stress response and reduced reproductive output in Yellow-eyed penguins exposed to unregulated tourism. *General and Comparative Endocrinology*, 152, 54-63

Fowler GS (1999) Behavioral and hormonal responses of Magellanic penguins (*Spheniscus magellanicus*) to tourism and nest site visitation. *Biological Conservation*, 90, 143-149

Gobush KS, Mutayoba BM, Wasser SK (2008) Long-term impacts of poaching on relatedness, stress physiology, and reproductive output of adult female African Elephants. *Conservation Biology*, 22, 1590-1599

Graham CM, Narayan EJ, McCallum H, Hero JM (2013) Non-invasive monitoring of glucocorticoid physiology within highland and lowland populations of native Australian Great Barred Frog (*Mixophyes fasciolatus*). *General and Comparative Endocrinology*, 191, 24–30

Hayward LS, Bowles A, Ha JC, Wasser SK (2011) Impacts of acute and long-term vehicle exposure on physiology and reproductive success of the northern spotted owl. *Ecosphere 2*, 1-20

Jaimez NA, Bribiescas RG, Aronsen GP, Anestis SA, Watts DP (2012) Urinary cortisol levels of gray-cheeked mangabeys are higher in disturbed compared to undisturbed forest areas in Kibale National Park, Uganda. *Animal Conservation*, 15, 242-247

Kelm DH, Popa-Lisseaunu AG, Dehnhard M, Ibáñez C (2015) Non-invasive monitoring of stress hormones in the bat *Eptesicus isabellinus* – Do fecal glucocorticoid metabolite concentrations correlate with survival? *General and Comparative Endocrinology*, 226, 27-35

Kindermann C, Narayan EJ, Hero JM (2012) Urinary corticosterone metabolites and chytridiomycosis disease prevalence in a free-living population of male Stony Creek frogs (*Litoria wilcoxii*). *Comparative Biochemistry and Physiology, Part A*, 162, 171–176

Kindermann C, Narayan EJ, Wild F, Wild CH, Hero JM (2013) The effect of stress and stress hormones on dynamic colour-change in a sexually dichromatic Australian frog. *Comparative Biochemistry and Physiology, Part A*, 165, 223–227

Kleist NJ, Guralnick RP, Cruz A, Lowry CA, Francis CD (2018) Chronic anthropogenic noise disrupts glucocorticoid signaling and has multiple effects on fitness in an avian community. *Proceedings of the National Academy of Sciences of the United States of America*, 115, E648–E657

Lèche A, Bazzano G, Hansen C, Navarro JL, Martin RH, Martella MB (2014) Stress in wild Greater Rhea populations *Rhea americana*: effects of agricultural activities on seasonal excreted glucocorticoid metabolite levels. *Journal of Ornithology*, 155, 919-926

Lewanzik D, Kelm DH, Greiner S, Dehnhard M, Voigt CC (2012) Ecological correlates of cortisol levels in two bat species with contrasting feeding habits. *General and Comparative Endocrinology*, 177, 104-112

Lunde ET, Bech C, Fyumagwa RD, Jackson CR, Røskaft E (2016) Assessing the effect of roads on impala (*Aepyceros melampus*) stress levels using faecal glucocorticoid metabolites. *African Journal of Ecology*, 54, 434-441

Malcolm KD, McShea WJ, Van Deelan WJ, Bacon HJ, Liu F, Putman F, Zhu X, Brown JL (2013) Analyses of fecal and hair glucocorticoids to evaluate short- and long-term stress and recovery of Asiatic black bears (*Ursus thibetanus*) removed from bile farms in China. *General and Comparative Endocrinology*, 185, 97-106

Morgan GM, Wilcoxen TE, Rensel MA, Schoech SJ (2012) Are roads and traffic sources of physiological stress for the Florida scrub-jay? *Wildlife Research,* 39, 301-310

Muehlenbein MP, Ancrenaz M, Sakong R, Ambu L, Prall S, et al. (2012) Ape Conservation Physiology: Fecal Glucocorticoid Responses in Wild Pongo *Pygmaeus morio* following Human Visitation. *PLoS ONE*, 7, e33357

Müllner A, Linsenmair KE, Wikelski M (2004) Exposure to ecotourism reduces survival and affects stress response in hoatzin chicks (*Opisthocomus hoazin*). *Biological Conservation*, 118, 549–558

Munerato MS, Marques JA, Caulkett NA, Tomás WM, Zanetti ES, Trovati RG, Pereira GT, Palme R (2015) Hormonal and behavioural stress responses to capture and radio-collar fitting in free-ranging pampas deer (*Ozotoceros bezoarticus*). *Animal Welfare*, 24, 437-446

Munshi-South J, Tchignoumba L, Brown J, Abbondanza N, Maldonado JE, Henderson A, Alonso A (2008) Physiological indicators of stress in African forest elephants (*Loxodonta africana cyclotis*) in relation to petroleum operations in Gabon, Central Africa. *Diversity and Distributions*, 14, 995-1003

Narayan EJ, Cockrem JF, Hero JM (2011) Urinary corticosterone metabolite responses to capture and captivity in the cane toad (*Rhinella marina*). *General and Comparative Endocrinology*, 173, 371–377

Narayan EJ, Molinia FC, Kindermann C, Cockrem JF, Hero JM (2011) Urinary corticosterone responses to capture and toe-clipping in the cane toad (*Rhinella marina*) indicate that toe-clipping is a stressor for amphibians. *General and Comparative Endocrinology*, 174, 238–245

Narayan EJ, Cockrem JF, Hero JM (2012) Effects of temperature on urinary corticosterone metabolite responses to short-term capture and handling stress in the cane toad (*Rhinella marina*). *General and Comparative Endocrinology*, 178, 301–305

Narayan EJ, Hero JM, Cockrem JF (2012) Inverse urinary corticosterone and testosterone metabolite responses to different durations of restraint in the cane toad (*Rhinella marina*). *General and Comparative Endocrinology*, 179, 345–349

Narayan EJ, Gramapurohit NP (2016) Sexual dimorphism in baseline urinary corticosterone metabolites and their association with body-condition indices in a peri-urban population of the common Asian toad (*Duttaphrynus melanostictus*). *Comparative Biochemistry and Physiology, Part A*, 191, 174–179

Newcomb Homan R, Regosin JV, Rodrigues DM, Reed JM, Windmiller BS, Romero LM (2003) Impacts of varying habitat quality on the physiological stress of spotted salamanders (*Ambystoma maculatum*). *Animal Conservation*, 6, 11-18

O'Brien JK, Steinman KJ, Fetter GA, Robeck TR (2017) Androgen and glucocorticoid production in the male killer whale (*Orcinus orca*): influence of age, maturity, and environmental factors. *Andrology*, 5, 180-190

Owen DAS, Carter ET, Holding ML, Islam K, Moore IT (2014) Roads are associated with a blunted stress response in a North American pit viper. *General and Comparative Endocrinology*, 202, 87–92

Ozella L, Anfossi L, Di Nardo F, Pessani D (2017) Effect of weather conditions and presence of visitors on adrenocortical activity in captive African penguins (*Spheniscus demersus*). *General and Comparative Endocrinology*, 242, 49–58

Partecke J, Schwabi I, Gwinner E (2006) Stress and the city: Urbanization and its effects on the stress physiology in European Blackbirds. *Ecology*, 87, 1945-1952

Piñeiro A, Narja I, Silván G, Illera JC (2012) Effects of tourist pressure and reproduction on physiological stress response in wildcats: management implications for species conservation. *Wildlife Research*, 39, 532–539

Rangel-Negrín A, Alfaro JL, Valdez RA, Romano MC, Serio-Silva JC (2009) Stress in Yucatan spider monkeys: effects of environmental conditions on fecal cortisol levels in wild and captive populations. *Animal Conservation*, 12, 496-502

Rimbach R, Link A, Heistermann M, Gómez-Posada C, Galvis N, Heymann EW (2013) Effects of logging, hunting, and forest fragment size on physiological stress levels of two sympatric ateline primates in Colombia. *Conservation Physiology,* 1, doi:10.1093/conphys/cot031.

Romero LM, Wikelski M (2002) Exposure to tourism reduces stress-induced corticosterone levels in Galápagos marine iguanas. *Biological Conservation*, 108, 371–374

Russ A, Reitemeier S, Weismann A, Gottschalk J, Einspanier A, Klenke R (2015) Seasonal and urban effects on the endocrinology of a wild passerine. *Ecology and Evolution*, 5, 5698–5710

Schoech SJ, Bowman R, Bridge ES, Boughton RK (2007) Baseline and acute levels of corticosterone in Florida Scrub-Jays (*Aphelocoma coerulescens*): Effects of food supplementation, suburban habitat, and year. *General and Comparative Endocrinology*, 154, 150-160

Seltmann A, Czirják GÁ, Courtiol A, Bernard H, Struebig MJ, Voigt CC (2017) Habitat disturbance results in chronic stress and impaired health status in forest-dwelling paleotropical bats. *Conservation Physiology*, 5, doi:10.1093/conphys/cox020.

Shutt K, Heistermann M, Kasin A, Todd A, Kalousova B, Profosouva I, Petrzelkova K, Fuh T (2014) Effects of habituation, research and ecotourism on faecal glucocorticoid metabolites in wild western lowland gorillas: Implications for conservation management. *Biological Conservation*, 172, 72-79

Spercoski KM, Morais RN, Morato RG, de Paula RC, Azevedo FC, May-Júnior JA, Santos JP, Reghelin AL, Wildt DE, Songsasen N (2012) Adrenal activity in maned wolves is higher on farmlands and park boundaries than within protected areas. *General and Comparative Endocrinology*, 179, 232-240

Strasser EH, Heath JA (2013) Reproductive failure of a human-tolerant species, the American kestrel, is associated with stress and human disturbance. *Journal of Applied Ecology*, 50, 912–919

Strochlic DE, Romero LM (2008) The effects of chronic psychological and physical stress on feather replacement in European starlings (*Sturnus vulgaris*). *Comparative Biochemistry and Physiology, Part A*, 149, 68–79

Tennessen JB, Parks SE, Langkilde T (2014) Traffic noise causes physiological stress and impairs breeding migration behaviour in frogs. *Conservation Physiology,* 2, doi:10.1093/conphys/cou032.

Thiel D, Jenni-Eiermann S, Braunisch V, Palme R, Jenni L (2008) Ski tourism affects habitat use and evokes a physiological stress response in capercaillie *Tetrao*

urogallus: a new methodological approach. *Journal of Applied Ecology*, 45, 845-853

Walker BG, Boersma PD, Wingfield JC (2006) Habituation of adult Magellanic penguins to human visitation as expressed through behaviour and corticosterone secretion. *Conservation Biology*, 20, 146–154

Walker BG, Boersma PD, Wingfield JC (2005) Physiological and behavioral differences in Magellanic penguin chicks in undisturbed and tourist-visited locations of a colony. *Conservation Biology*, 19, 1571-1577

Washburn BE, Millspaugh JJ (2002) Effects of simulated environmental conditions on glucocorticoid metabolite measurements in white-tailed deer feces. *General and Comparative Endocrinology*, 127, 217–222

Wasserman MD, Chapman CA, Milton K, Goldberg TL, Ziegler TE (2013) Physiological and behavioral effects of capture darting on Red Colobus monkeys (*Procolobus rufomitratus*) with a comparison to Chimpanzee (*Pan troglodytes*) predation. *International Journal of Primatology*, 34, 1020-1031

Webster K, Narayan EJ, de Vos N (2017) Fecal glucocorticoid metabolite response of captive koalas (*Phascolarctos cinereus*) to visitor encounters. *General and Comparative Endocrinology*, 244, 157–163

Wielebnowski NC, Fletchal N, Carlstead K, Busso JM, Brown JL (2002) Noninvasive assessment of adrenal activity associated with husbandry and behavioural factors in the North American Clouded Leopard Population. *Zoo Biology*, 21, 77-98

Appendix 4.2 Systematic review of the literature pertaining to the impact of roads on glucocorticoids in wild living animals

A total of 500 studies were assessed for relevance in two databases (Google Scholar and Web of Science). Studies were selected if they investigated the impact of at least one road related variable on glucocorticoids in free living animals. Studies on captive animals were excluded as were studies that examined the effect of roads on other stress related hormones such as catecholamines.

In Google Scholar, the first three hundred titles retrieved with the search terms “stress OR stress hormones OR glucocorticoids OR cortisol OR corticosterone AND roads OR traffic OR vehicles OR motorway AND animals OR conservation” were checked for relevant studies.

In *Web of Science*, the first two hundred titles retrieved with the search terms “stress OR stress hormones OR cortisol OR corticosterone OR glucocorticoids AND roads OR traffic AND conservation” were checked for relevant studies.

Additional studies were then identified from the references of these studies.

References in Table 4.2

[1] Baxter-Gilbert JH, Riley JL, Mastromonaco GF, Litzgus JD, Lesbarrères D (2014) A novel technique to measure chronic levels of corticosterone in turtles living around a major roadway. *Conservation Physiology,* 2: doi: 10. 1093/conphys/cou036

[2] Blickley JL, Word KR, Krakauer AH, Phillips JL, Sells AN, Taff CC, Wingfield JC, Patricelli GL (2012) Experimental chronic noise is related to elevated fecal corticosteroid metabolites in lekking male Greater Sage-Grouse (*Centrocercus urophasianus*). *PLoS ONE,* 7(11): e50462. Doi: 10.1371/journal.pone.0050462

[3] Brearley G, McAlpine C, Bell S, Bradley A (2012) Influence of urban edges on stress in an arboreal mammal: a case study of squirrel gliders in southeast Queensland, Australia. *Landscape Ecology,* 27, 1407-1419

[4] Creel S, Fox JE, Hardy A, Sands J, Garrott B, Peterson RO (2002) Snowmobile activity and glucocorticoid stress responses in wolves and elk. *Conservation Biology,* 16, 809-814

[5] Crino OL, Klaassen Van Oorschot B, Johnson EE, Malisch JL, Breuner CW (2011) Proximity to a high traffic road: Glucocorticoid and life history consequences for nestling white-crowned sparrows. *General and Comparative Endocrinology*, 173, 323-332

[6] Hayward LS, Bowles AE, Ha JC, Wasser SK (2011) Impacts of acute and long-term vehicle exposure on physiology and reproductive success of the northern spotted owl. *Ecosphere*, 2(6): art65. Doi: 10.1890/ES10-00199.1

[7] Lunde ET, Bech C, Fyumagwa RD, Jackson CR, Røskaft E (2016) Assessing the effect of roads on impala (*Aepyceros melampus*) stress levels using faecal glucocorticoid metabolites. *African Journal of Ecology*, 54, 434-441

[8] Morgan GM, Wilcoxen TE, Rensel MA, Schoech AJ (2012) Are roads and traffic sources of physiological stress for the Florida scrub-jay? *Wildlife Research*, 39, 301-310

[9] Owen DAS, Carter ET, Holding ML, Islam K, Moore IT (2014) Roads are associated with blunted stress response in a North American pit viper. *General and Comparative Endocrinology*, 202, 87-92.

[10] Strasser EH, Heath JA (2013) Reproductive failure of a human-tolerant species, the American kestrel, is associated with stress and human disturbance. *Journal of Applied Ecology*, 50, 912-919.

[11] Tempel DJ, Gutiérrez RJ (2004) Factors related to fecal corticosterone levels in California Spotted Owls: Implications for assessing chronic stress. *Conservation Biology*, 18, 538-547.

[12] Tennessen JB, Parks SE, Langkilde T (2014) Traffic noise causes physiological stress and impairs breeding migration behaviour in frogs. *Conservation Physiology*, 2: doi: 10.1093/conphys/cou032

[13] Wasser AK, Bevis K, King G, Hanson E (1997) Noninvasive physiological measures of disturbance in the Northern Spotted Owl. *Conservation Biology*, 11, 1019-1022

[14] Navarro-Castilla A, Mata C, Ruiz-Capillas P, Palme R, Malo JE, Barja I (2014) Are motorways potential stressors of roadside Wood Mice (*Apodemus sylvaticus*) populations? *PLoS ONE*, 9(3): e91942. doi:10.1371/journal.pone.0091942

[15] Cayuela H, Quay L, Dumet A, Léna JP, Miaud C, Rivière V (2015) Intensive vehicle traffic impacts morphology and endocrine stress response in a threatened amphibian. *Oryx*, 51, 182-188.

[16] Crino OL, Johnson EE, Blickley JL, Patricelli GL, Breuner CW (2013) Effects of experimentally elevated traffic noise on nestling white-crowned sparrow stress physiology, immune function and life history. *The Journal of Experimental Biology*, 216, 2055-2062.

[17] Angelier F, Meillère A, Grace JK, Trouvé C, Brischoux F (2015) No evidence for an effect of traffic noise on the development of the corticosterone stress response in an urban exploiter. *General and Comparative Endocrinology*, 232, 43-50.

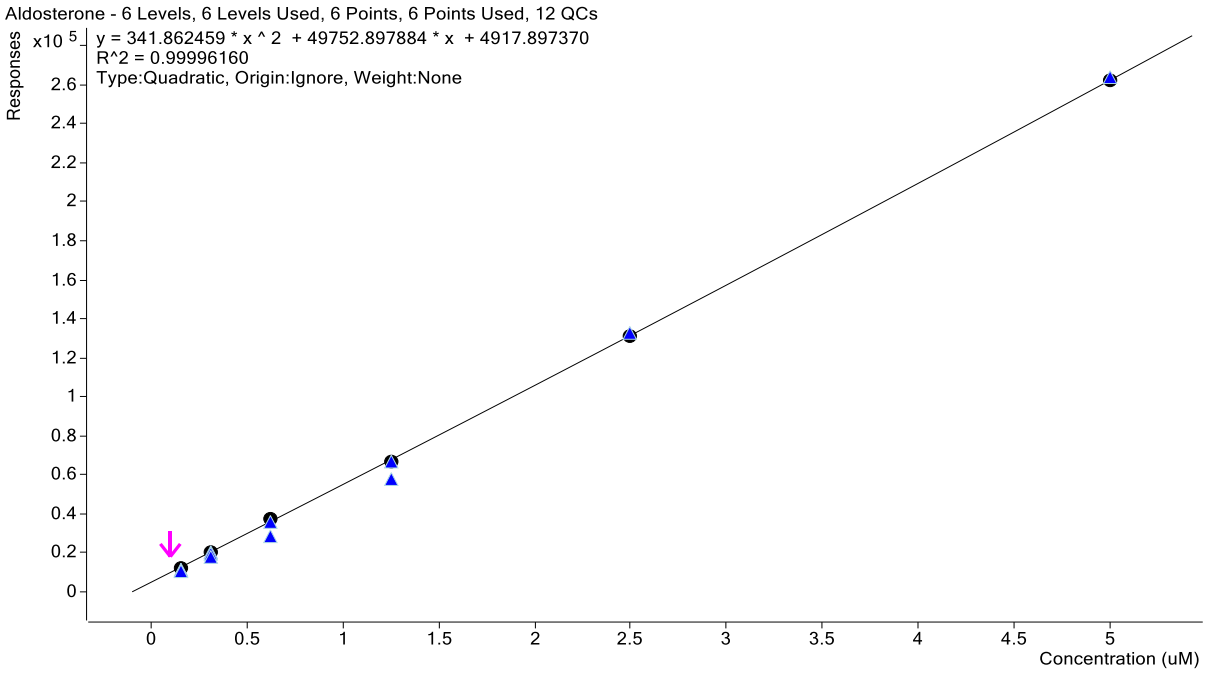
[18] Russ A, Reitemeier S, Weissmann A, Gottschalk J, Einspanier A, Klenke R (2015) Seasonal and urban effects on the endocrinology of a wild passerine. *Ecology and Evolution*, 5, 5698-5710.

Appendix 4.3 A complete list of target compounds including those not identified

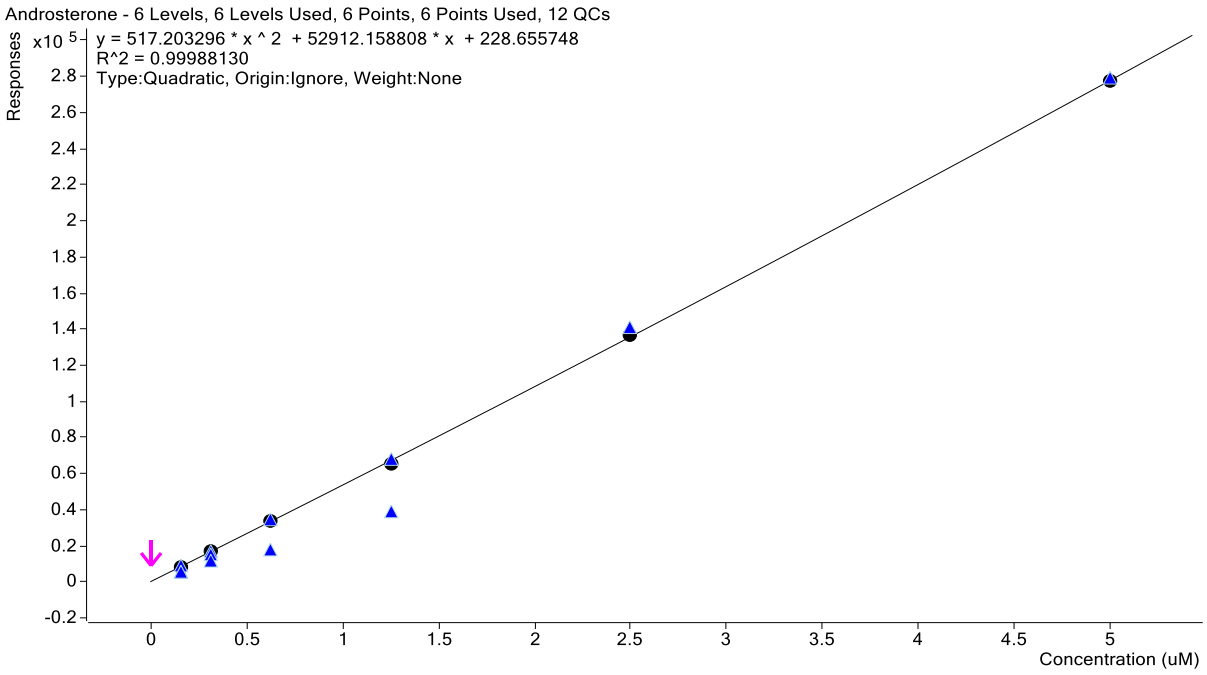
	Compound	Formula	Precursor m/z	ID Product m/z	Qualifier Product m/z	Fragmentor Voltage	Collision Energy Voltage (ID; Qual)	Approx RT
1	Testosterone	C19H28O2	289.2	109.1	97.1	100	25; 29	13.6
2	Progesterone	C21H30O2	315.2	109.1	97.1	80	25; 29	16.1
3	Estrone	C18H22O2	271.2	253.1	133.1	120	25; 9	13.0
4	17β-Estradiol	C18H24O2	273.2	107.1	135.1	80	35; 13	13.1
5	Pregnenolone	C21H32O2	317.3	299.3	159.1	80	9; 25	17.1
6	1-dehydro-testosterone	C19H26O2	287.2	121.0	135.1	80	25; 13	12.2
7	Dihydrotestost-erone (DHT)	C19H30O2	291.2	255.2	159.1	140	13; 25	15.3
8	Cortisone	C21H28O5	361.2	163.1	121.1	120	25; 35	9.0
9	Cortisol (Hydrocortisone)	C21H30O5	363.2	121.1	309.00	80	25; 15	9.8
10	Aldosterone	C21H28O5	361.2	343.2	315.2	80	17; 21	8.1
11	Corticosterone	C21H30O4	347.2	121.0	329.2	100	25; 13	11.5
12	Androsterone	C19H30O2	291.2	255.2	273.2	80	13; 9	16.6
13	Estriol	C18H24O3	289.2	107.1	253.1	140	25; 9	7.6
14	11deoxy-corticosterone	C21H30O3	331.2	97.1	109.1	100	25; 29	13.3
15	17alpha-hydroxy-progesterone	C21H30O3	331.2	97.1	109.1	120	25; 35	14.1
	Umbelliferone	C9H6O3	163.0	107.1	77.1	120	25; 35	4.2

Appendix 4.4 Calibration curves for aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j) and β-estradiol (k).

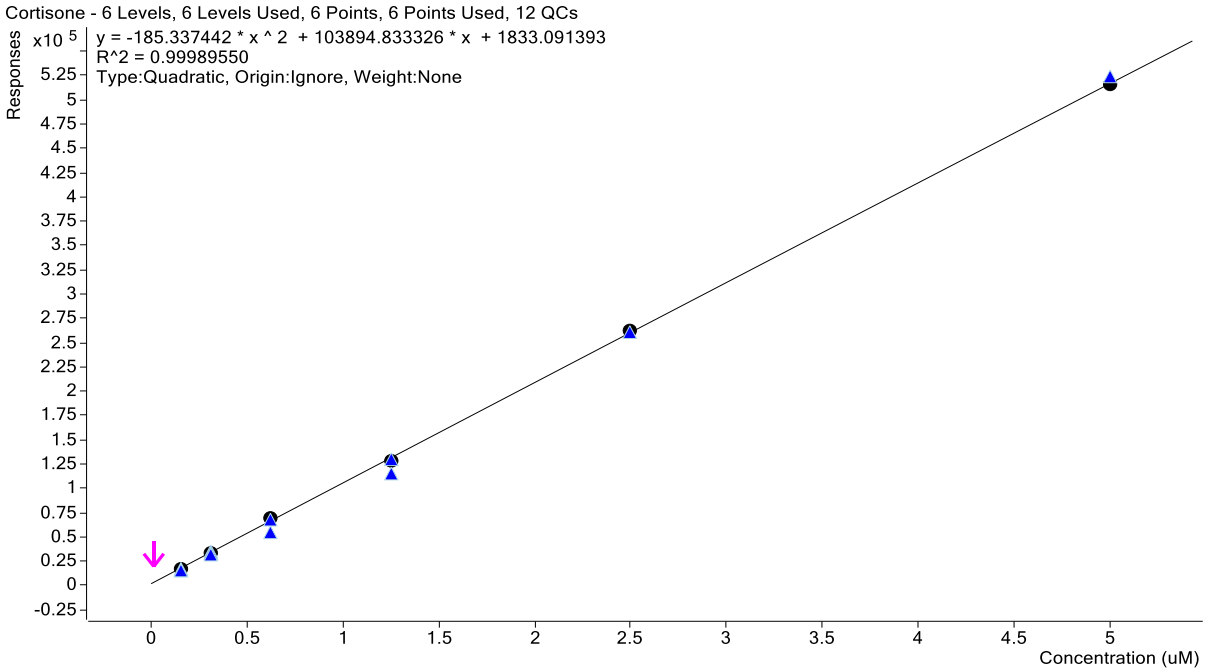
(a)



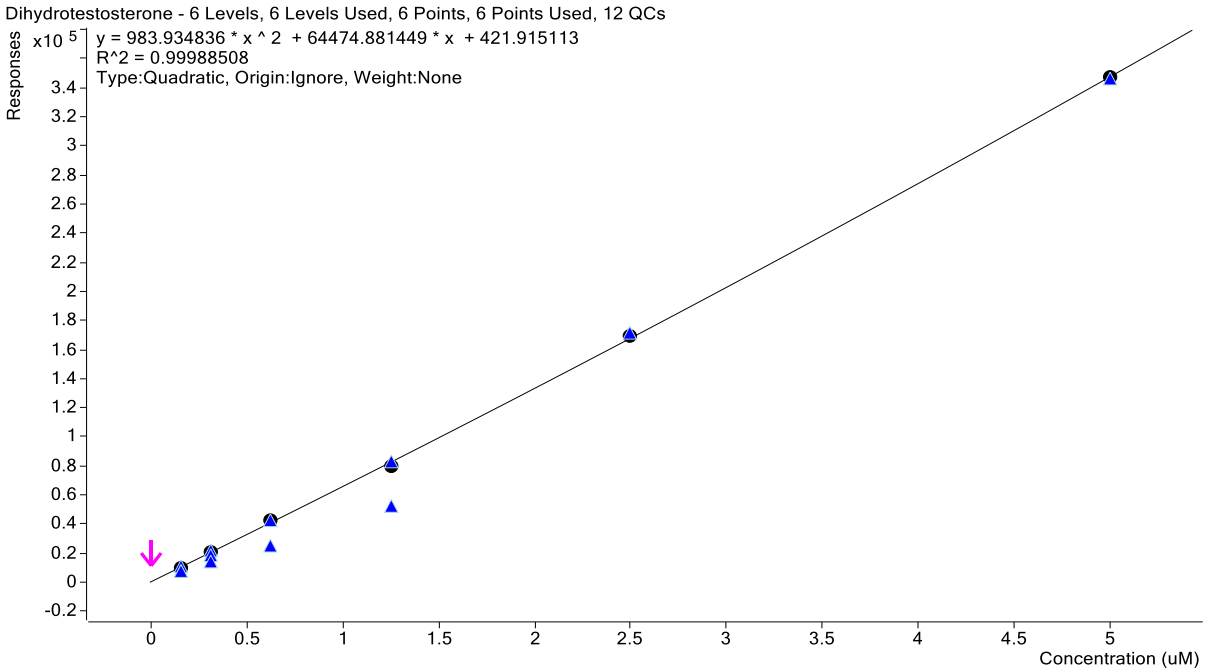
(b)



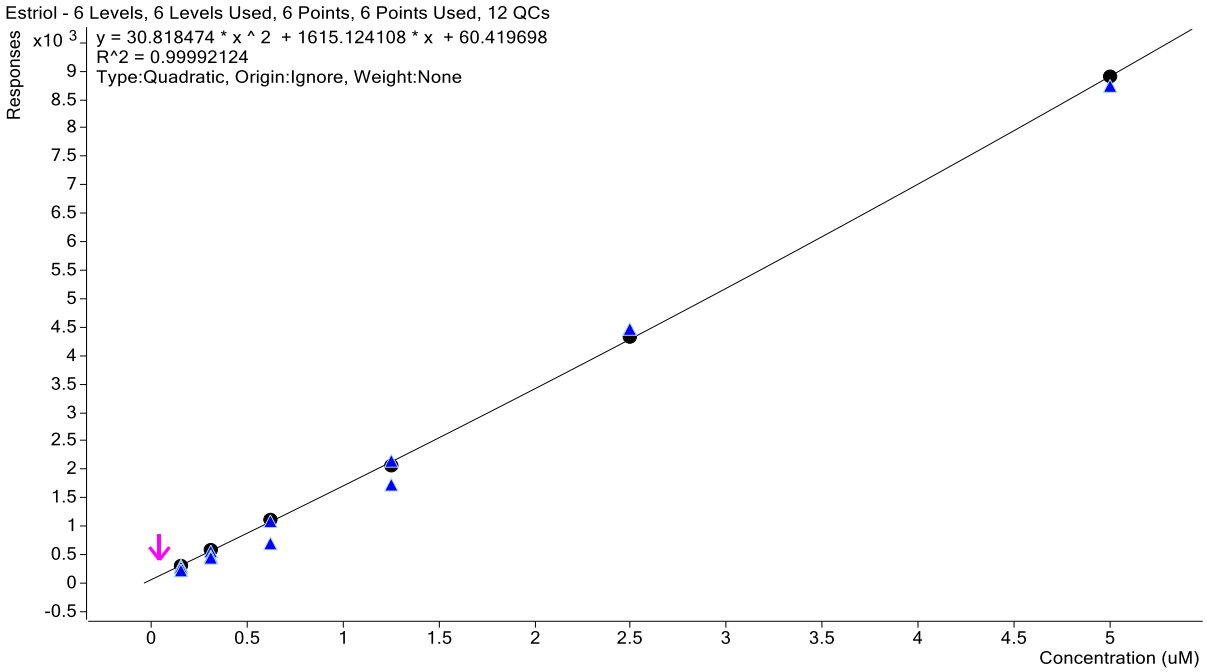
(c)


Cortisone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = -185.337442 * x ^ 2 + 103894.833326 * x + 1833.091393
R^2 = 0.99989550
Type:Quadratic, Origin:Ignore, Weight:None
Responses
x10 5
Concentration (uM)

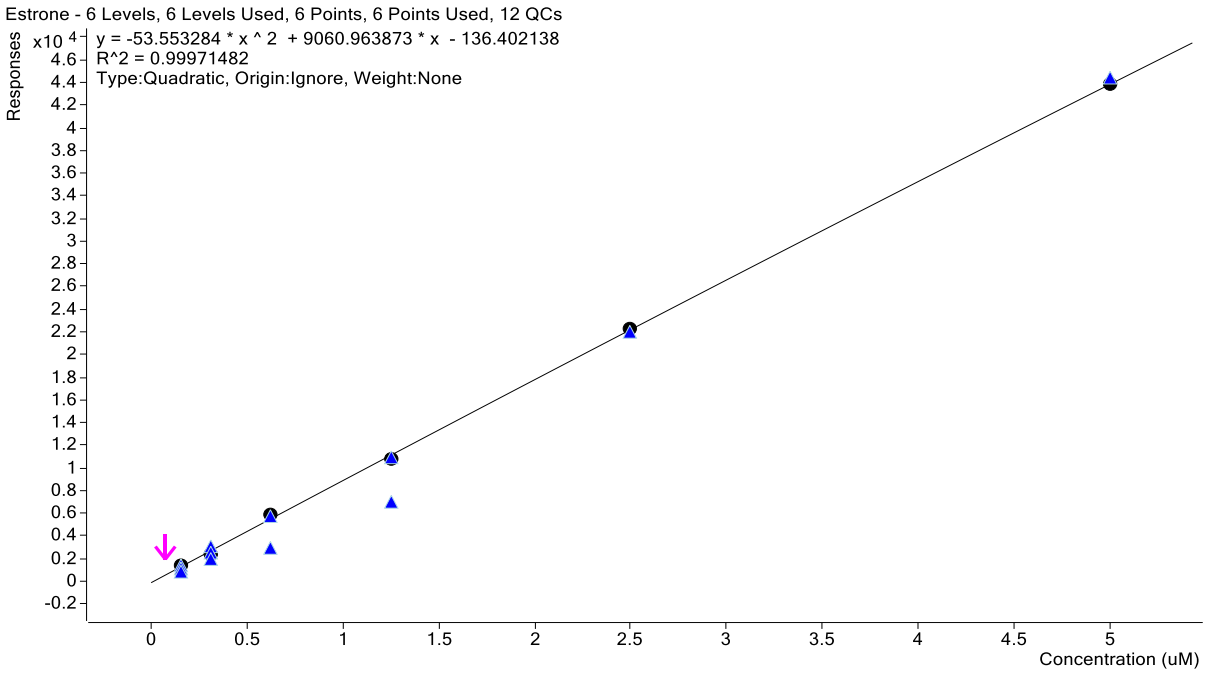
(d)


Dihydrotestosterone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = 983.934836 * x ^ 2 + 64474.881449 * x + 421.915113
R^2 = 0.99988508
Type:Quadratic, Origin:Ignore, Weight:None
Responses
x10 5
Concentration (uM)

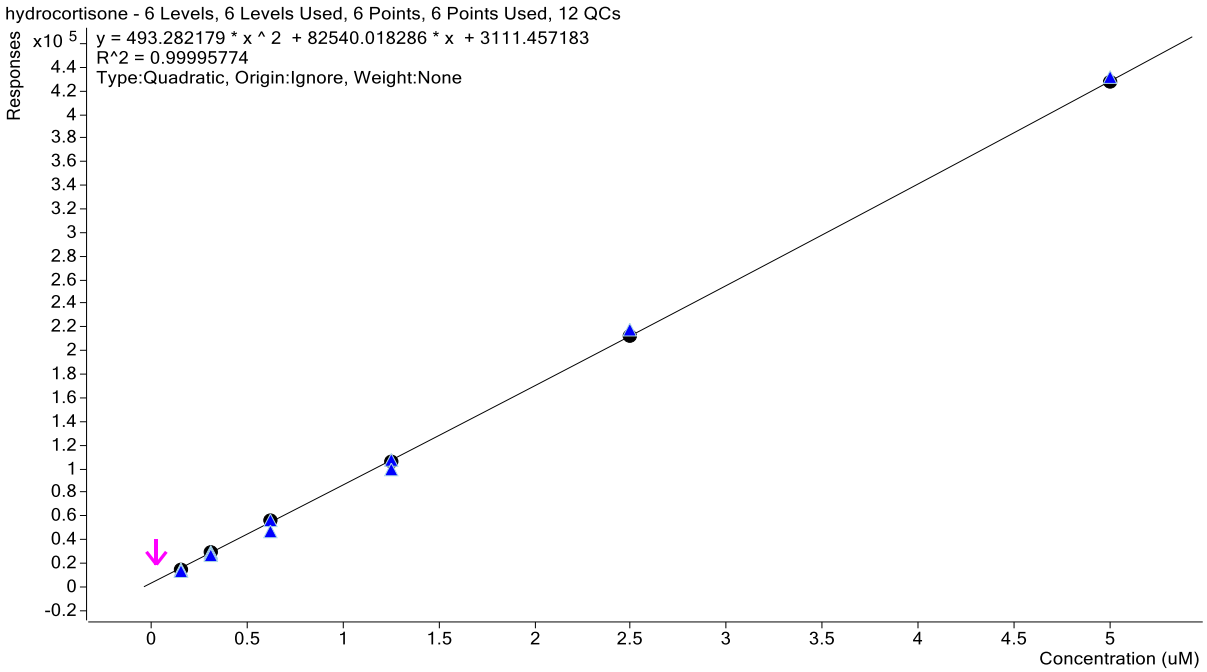
(e)


Estriol - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = 30.818474 * x ^ 2 + 1615.124108 * x + 60.419698
R^2 = 0.99992124
Type:Quadratic, Origin:Ignore, Weight:None
Responses
x10 3
Concentration (uM)

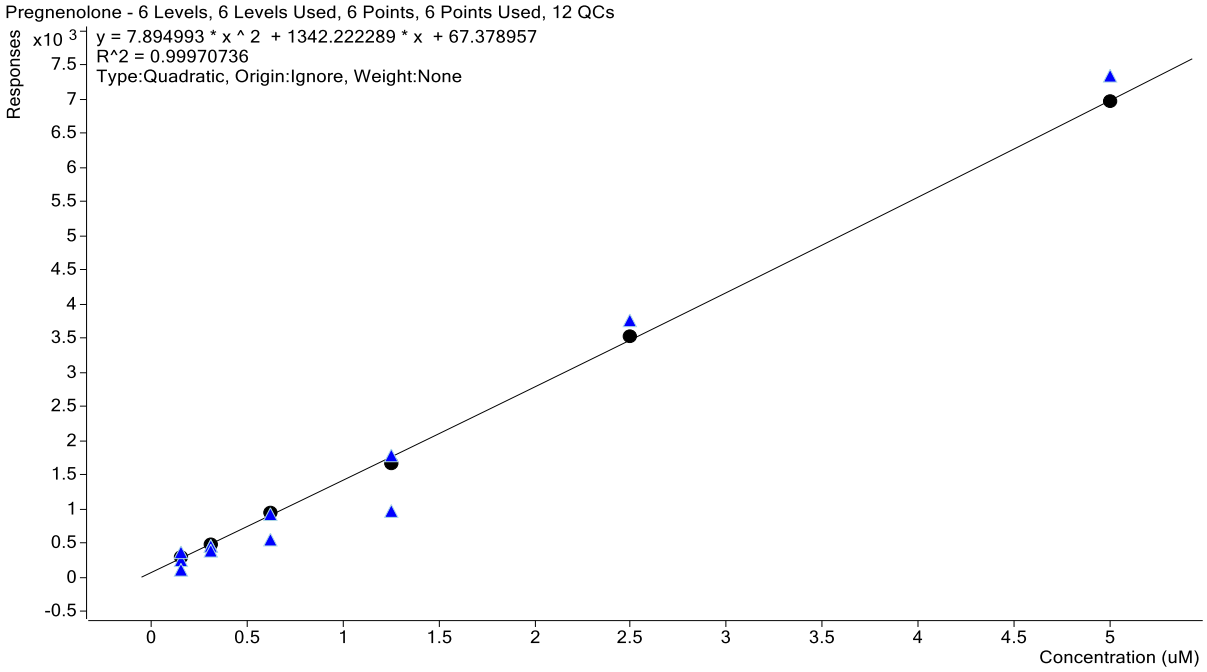
(f)


Estrone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = -53.553284 * x ^ 2 + 9060.963873 * x - 136.402138
R^2 = 0.99971482
Type:Quadratic, Origin:Ignore, Weight:None
Responses
x10 4
Concentration (uM)

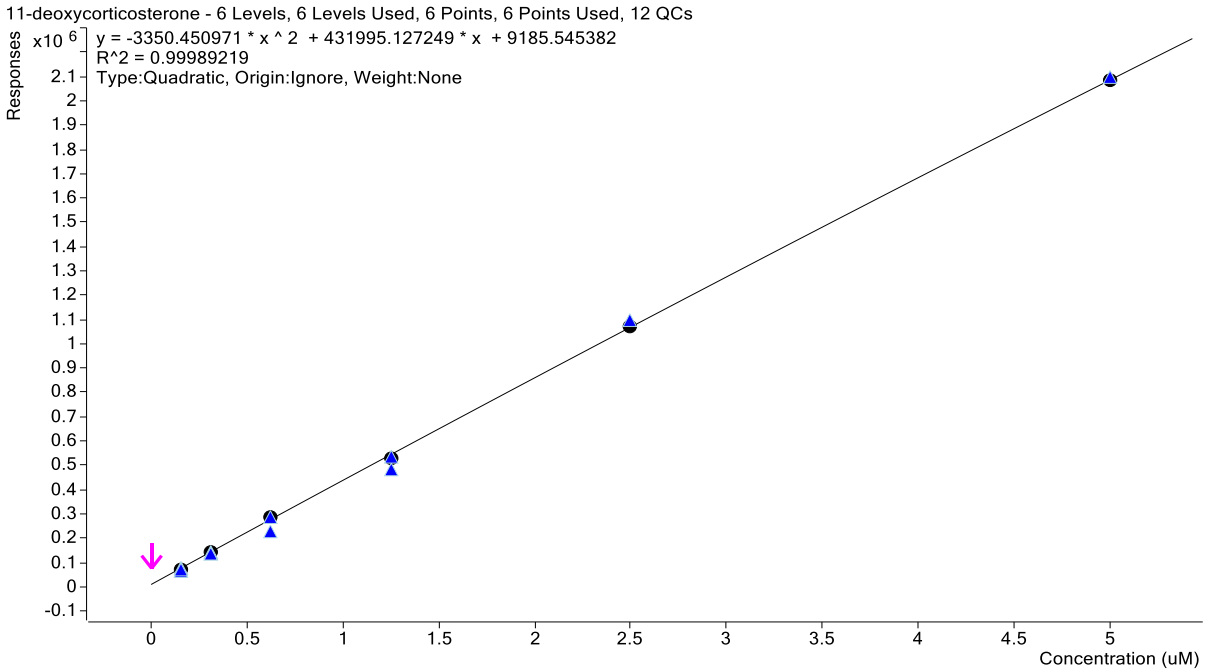
(g)


hydrocortisone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = 493.282179 * x ^ 2 + 82540.018286 * x + 3111.457183
R^2 = 0.99995774
Type:Quadratic, Origin:Ignore, Weight:None
Responses
x10 5
Concentration (uM)

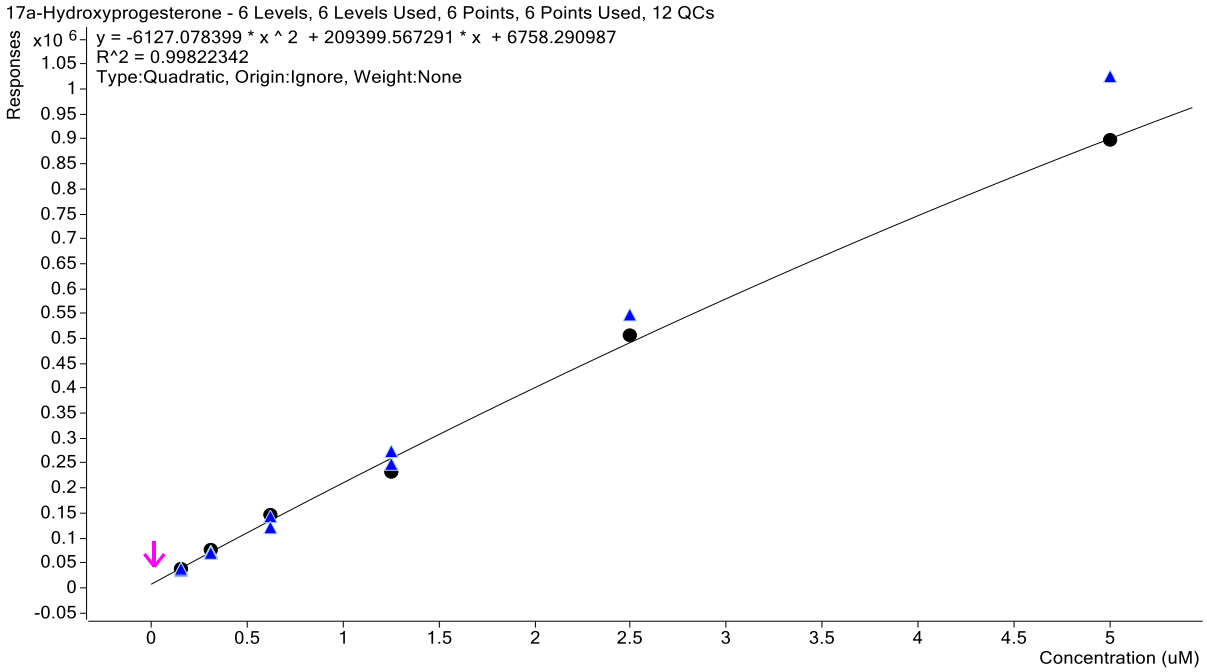
(h)


Pregnenolone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = 7.894993 * x ^ 2 + 1342.222289 * x + 67.378957
R^2 = 0.99970736
Type:Quadratic, Origin:Ignore, Weight:None
Responses
x10 3
Concentration (uM)

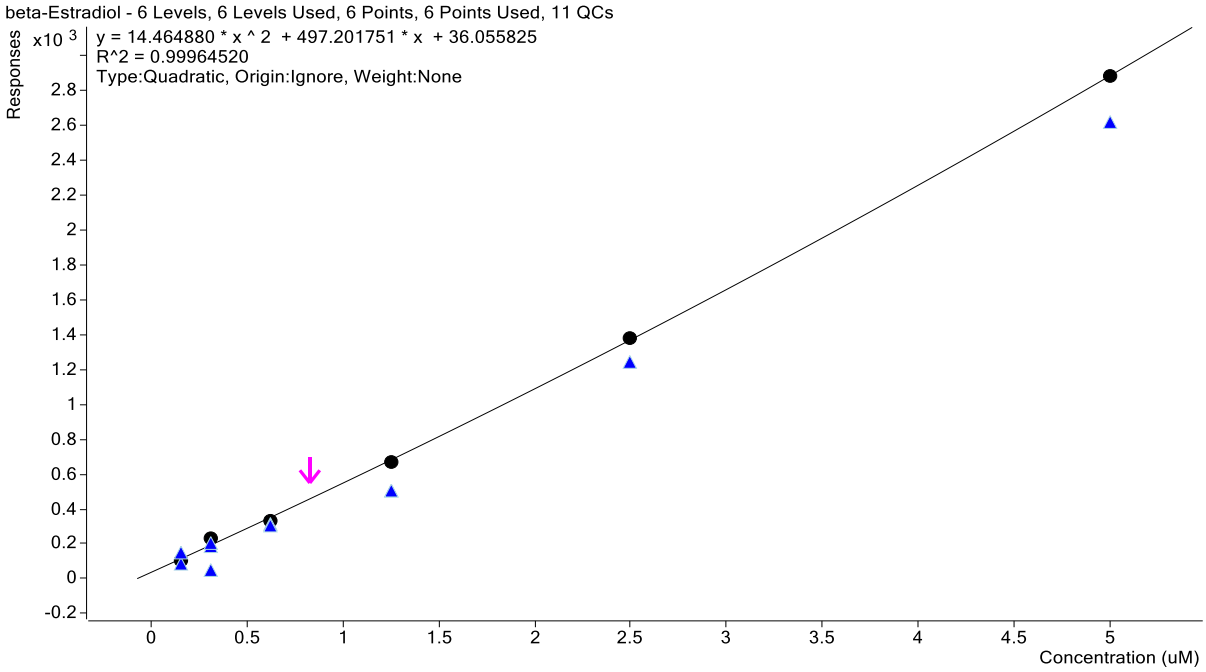
(i)


11-deoxycorticosterone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = -3350.450971 * x ^ 2 + 431995.127249 * x + 9185.545382
R^2 = 0.99989219
Type:Quadratic, Origin:Ignore, Weight:None
Responses
x10 6
Concentration (uM)

(j)


17a-Hydroxyprogesterone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = -6127.078399 * x ^ 2 + 209399.567291 * x + 6758.290987
R^2 = 0.99822342
Type:Quadratic, Origin:Ignore, Weight:None
Responses
x10 6
Concentration (uM)

(k)

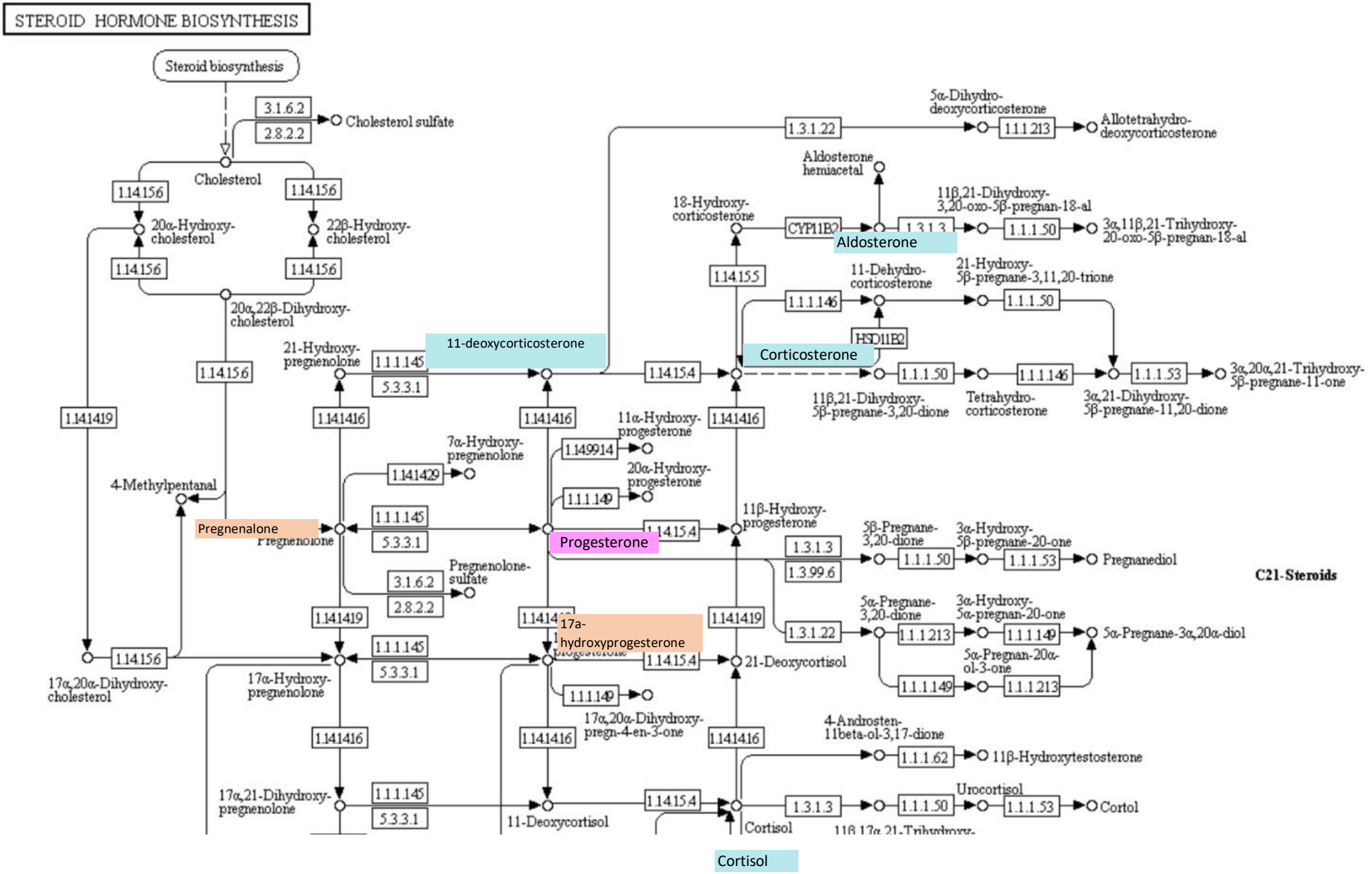

beta-Estradiol - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 11 QCs
y = 14.464880 * x ^ 2 + 497.201751 * x + 36.055825
R^2 = 0.99964520
Type:Quadratic, Origin:Ignore, Weight:None
Responses
x10 3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
Concentration (uM)

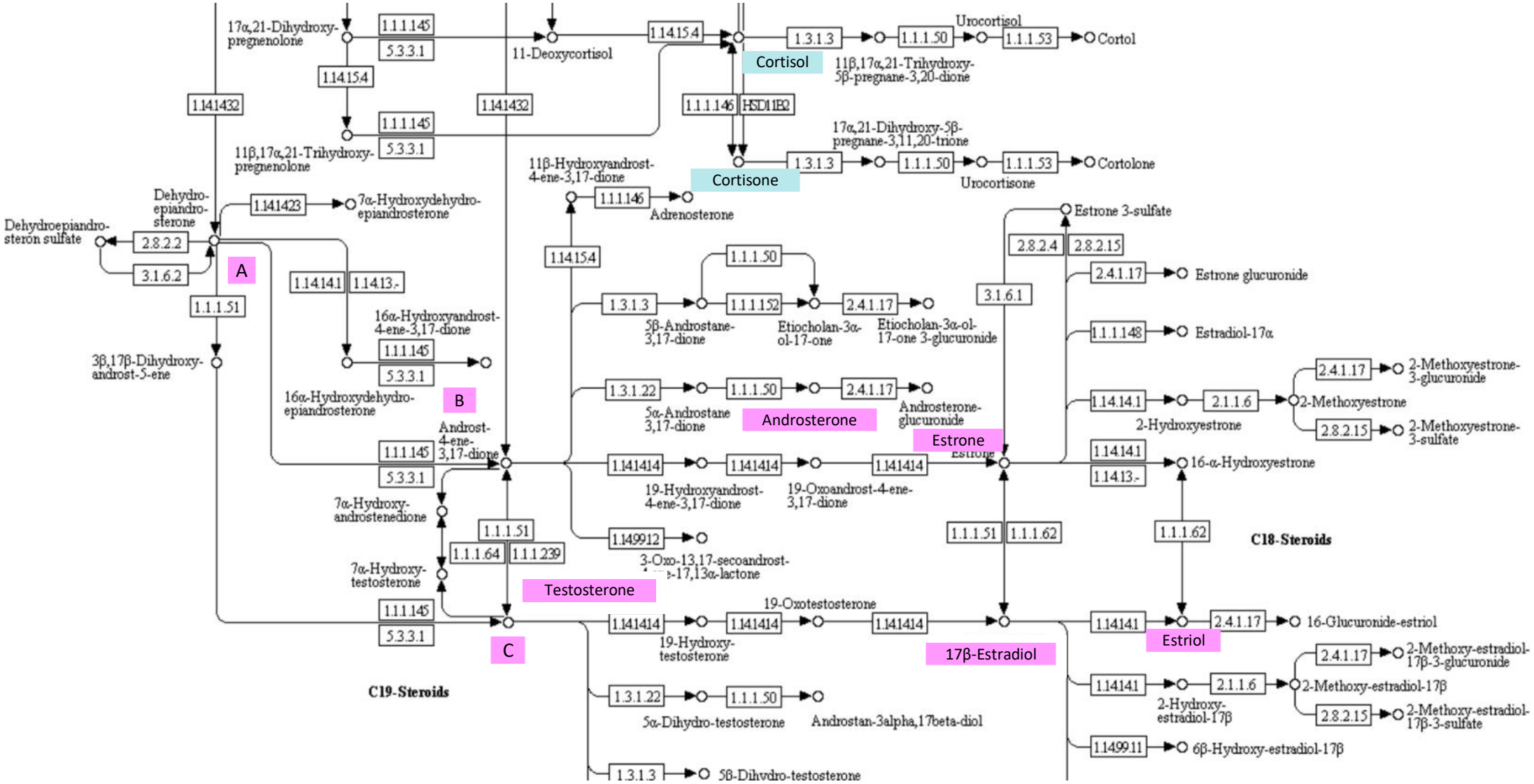
Appendix 4.5 The % relative standard error of samples.

%RSE	Estriol (RT7.62)	Aldosterone (RT8.13)	Cortisone (RT8.97)	hydrocortisone (RT9.82)	Estrone (RT12.98)	beta-Estradiol (RT13.04)	11-deoxycorticosterone (RT13.25)	17a-Hydroxyprogesterone (RT14.08)	Dihydrotestosterone (RT15.27)	Androsterone (RT16.61)	Pregnenolone (RT17.09)
1	77.65	5.51	5.43	11.64	68.97	60.58		15.52		22.36	3.97
2	74.62	8.52	29.11	64.51	27.22	84.50		20.18		31.76	35.33
3	61.42	13.19	55.50		71.18	76.37		11.83		40.98	39.90
4		9.66	58.51	68.17	29.03	41.42		28.37		57.22	26.32
5		1.71	15.23	6.53	37.39	44.80		23.63		80.24	9.85
6	87.98	7.87	3.60	100.00	8.71	12.55	7.41	15.93		100.00	26.13
7	59.46	5.90	8.24	15.33	9.70	48.41	81.37	51.41	100.00	86.53	69.92
8	100.00	14.20	16.94		32.16	47.34	10.51	43.98	100.00	23.91	21.52
9	100.00	3.02	4.60		28.18	45.76		19.05	100.00	50.11	5.20
10	57.52	7.67	9.41		32.23	35.54		16.43		70.62	7.13
11	100.00	5.10	7.36		23.88	53.42		19.79	51.28	57.44	32.17
12	100.00	6.58	3.16		34.27	49.56		11.76	53.93	31.95	30.65
13	78.87	8.47	6.83	16.88	4.72	27.49		20.39	76.55	62.15	38.86
14	100.00	13.67	13.04	24.77	34.25	68.76		31.20	87.15	100.00	54.03
15	100.00	9.38			26.53	30.76		6.38	40.36	8.50	27.12
16	50.94	18.21			36.20	36.34		8.31	34.68	4.72	36.50
17	51.23	8.10	100.00		21.30	42.84		5.43	100.00	10.57	13.71
18	23.48	5.39			18.03	12.69		7.77	33.58	14.91	20.40
19	12.95	3.99			18.86	6.83		14.37	18.21	6.15	9.61
20	26.80	8.46	1.29	6.00	41.95	27.94	5.94	31.31		100.00	14.25
21	1.32	6.47	3.50		13.56	3.96	5.35	36.54	100.00	100.00	65.27
22	12.59	10.46	11.22	6.51	33.95	8.08	26.57	19.71	88.16	100.00	15.36

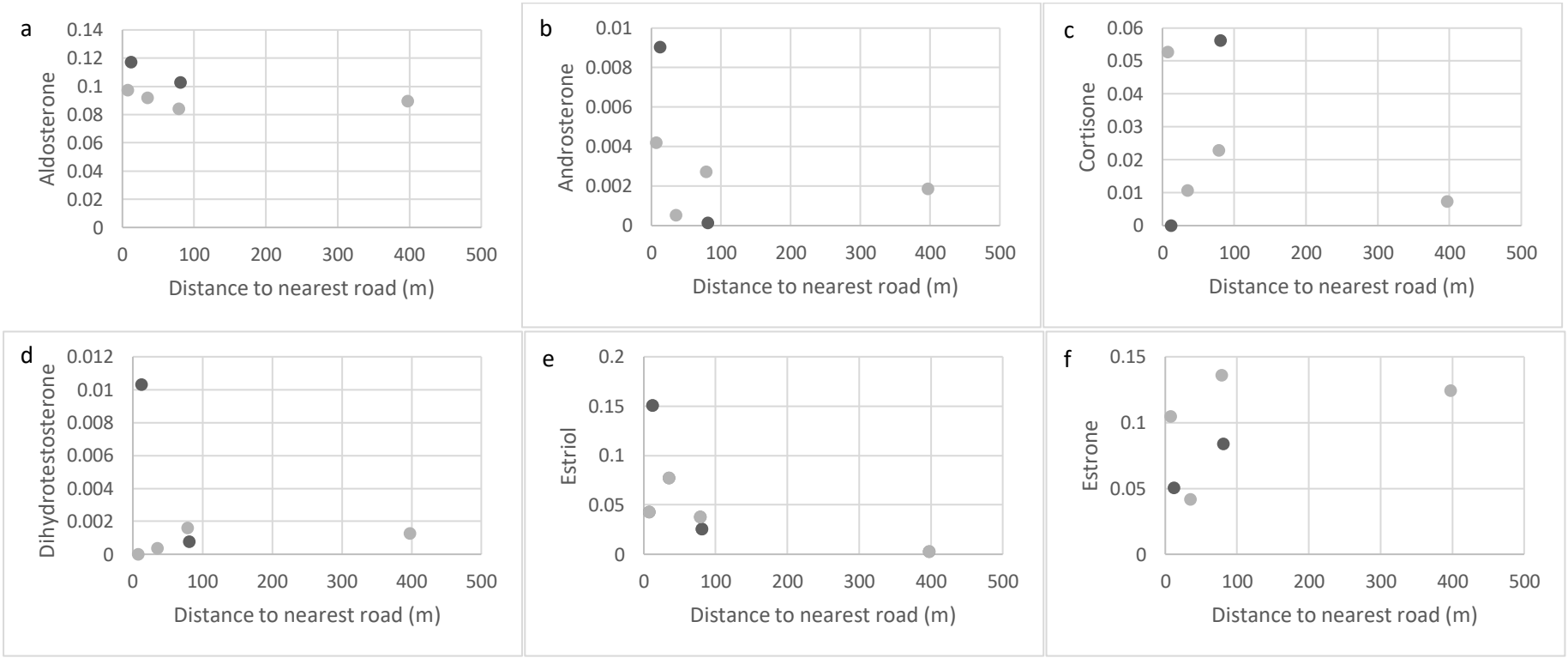
Appendix 4.6 (following page) The bionsynthesis pathway of steroid hormones. Target compounds are highlighted; reproductive hormones and their metabolites or precursors are highlighted in pink, corticosteroids and their precursors are highlighted in blue and precursors to both reproductive hormones and corticosteroids are highlighted in orange. Points A, B and C mark shared precursors (dehydroepiandrosterone, androst-4-ene-3,17-dione and testosterone respectively) of androsterone, estriol, dihydrotestosterone and 17β-estradiol.

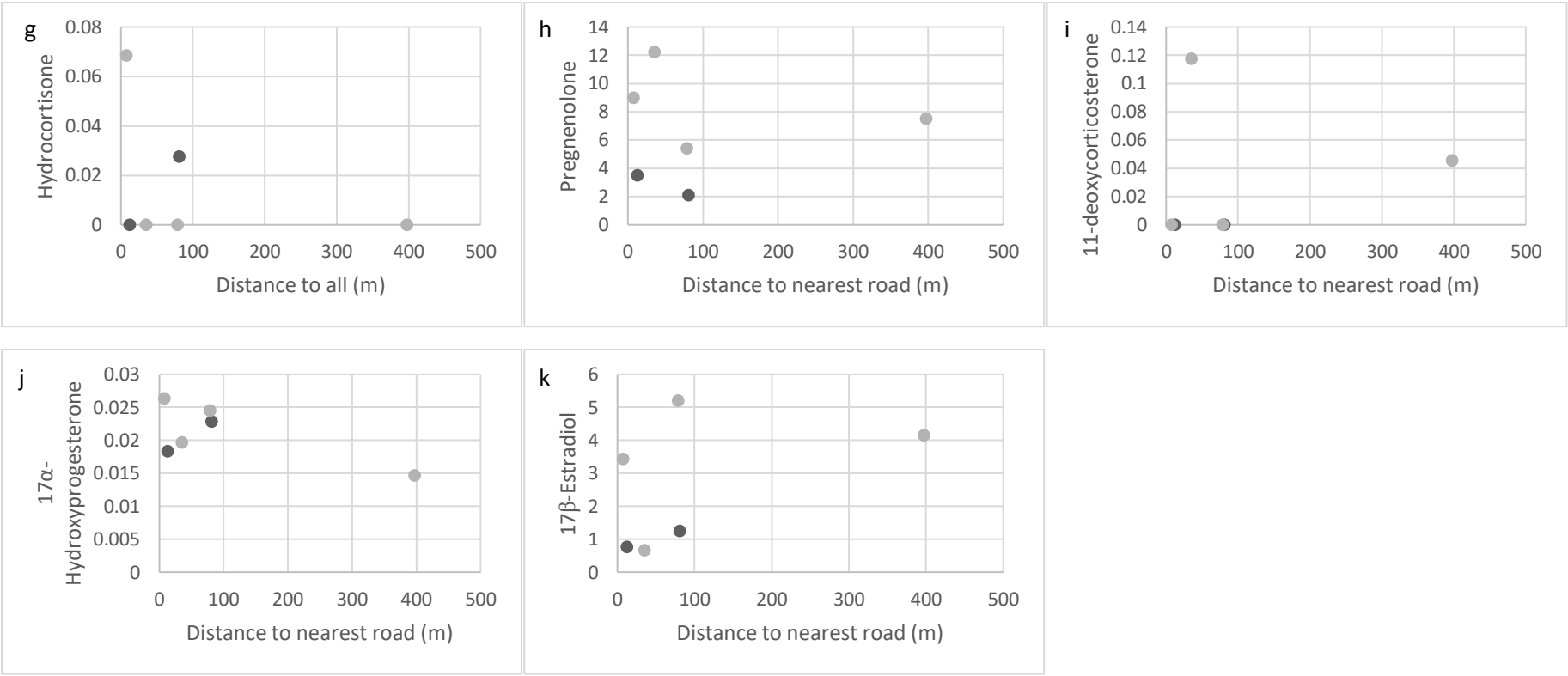
Appendix 4.6


STEROID HORMONE BIOSYNTHESIS
Steroid biosynthesis
Cholesterol
Cholesterol sulfate
20α-Hydroxy-cholesterol
22β-Hydroxy-cholesterol
20α,22β-Dihydroxy-cholesterol
21-Hydroxy-pregnenolone
11-deoxycorticosterone
Corticosterone
5α-Dihydro-deoxycorticosterone
Allotetrahydro-deoxycorticosterone
Aldosterone hemiacetal
18-Hydroxy-corticosterone
CYP11B2
Aldosterone
11β,21-Dihydroxy-3,20-oxo-5β-pregnan-18-al
3α,11β,21-Trihydroxy-20-oxo-5β-pregnan-18-al
11-Dehydro-corticosterone
21-Hydroxy-5β-pregnane-3,11,20-trione
HSD11B2
11β,21-Dihydroxy-5β-pregnane-3,20-dione
Tetrahydro-corticosterone
3α,21-Dihydroxy-5β-pregnane-11,20-dione
3α,20α,21-Trihydroxy-5β-pregnane-11-one
11α-Hydroxy-progesterone
7α-Hydroxy-pregnenolone
20α-Hydroxy-progesterone
4-Methylpentanal
Pregnenalone
Progesterone
11β-Hydroxy-progesterone
5β-Pregnane-3,20-dione
3α-Hydroxy-5β-pregnane-20-one
Pregnanediol
Pregnenolone-sulfate
17a-hydroxyprogesterone
5α-Pregnane-3,20-dione
3α-Hydroxy-5α-pregnan-20-one
5α-Pregnane-3α,20α-diol
5α-Pregnan-20α-ol-3-one
17α,20α-Dihydroxy-cholesterol
17α-Hydroxy-pregnenolone
21-Deoxycortisol
17α,20α-Dihydroxy-pregn-4-en-3-one
4-Androsten-11beta-ol-3,17-dione
11β-Hydroxytestosterone
17α,21-Dihydroxy-pregnenolone
11-Deoxycortisol
Cortisol
Urocortisol
Cortol
C21-Steroids
Cortisol

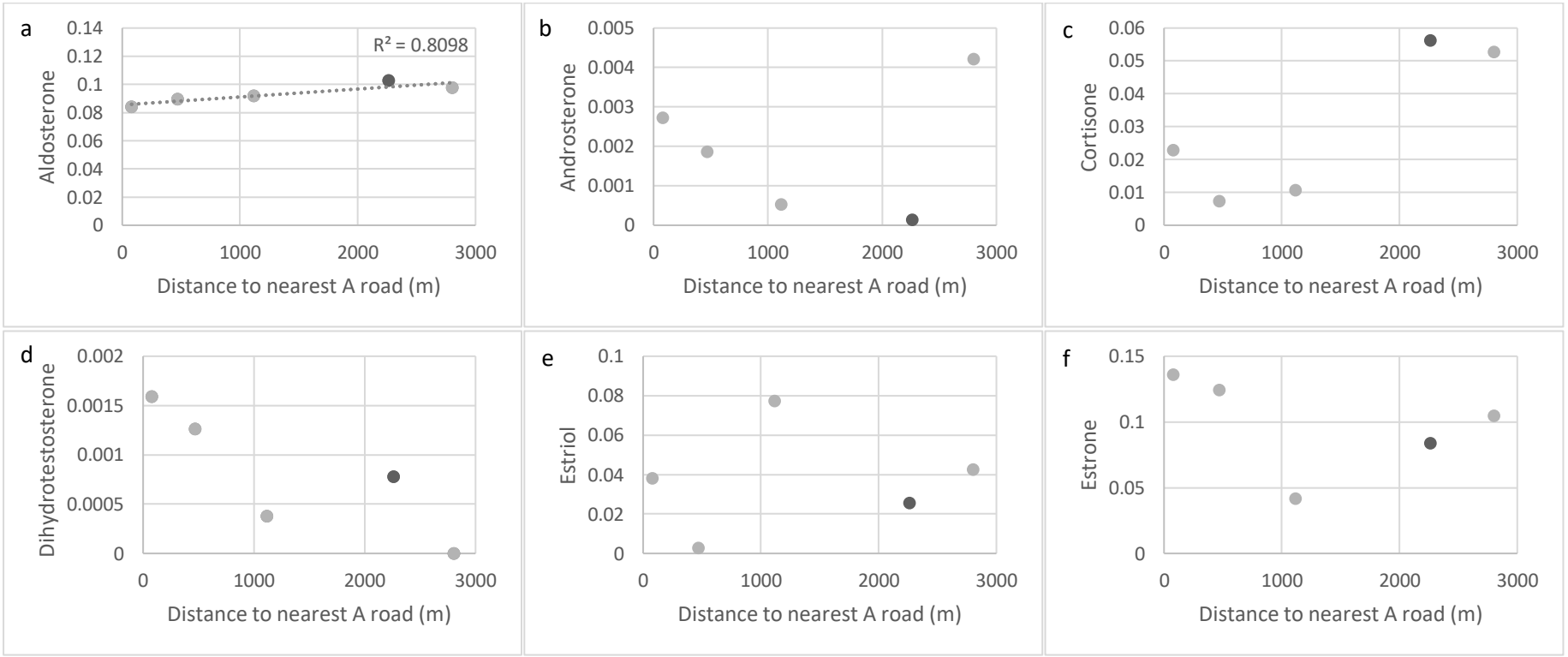

17α,21-Dihydroxy-pregnenolone
11-Deoxycortisol
Cortisol
Urocortisol
Cortol
11β,17α,21-Trihydroxy-5β-pregnane-3,20-dione
11β,17α,21-Trihydroxy-pregnenolone
17α,21-Dihydroxy-5β-pregnane-3,11,20-trione
Cortisone
Cortolone
Urocortisone
11β-Hydroxyandrost-4-ene-3,17-dione
Adrenosterone
Dehydro-epiandro-sterone
Dehydroepiandro-steron sulfate
7α-Hydroxydehydro-epiandrosterone
A
16α-Hydroxyandrost-4-ene-3,17-dione
3β,17β-Dihydroxy-androst-5-ene
16α-Hydroxydehydro-epiandrosterone
B
Androst-4-ene-3,17-dione
5β-Androstane-3,17-dione
Etiocholan-3α-ol-17-one
Etiocholan-3α-ol-17-one 3-glucuronide
5α-Androstane 3,17-dione
Androsterone
Androsterone-glucuronide
Estrone
Estrone 3-sulfate
Estrone glucuronide
Estradiol-17α
2-Hydroxyestrone
2-Methoxyestrone
2-Methoxyestrone-3-glucuronide
2-Methoxyestrone-3-sulfate
16-α-Hydroxyestrone
19-Hydroxyandrost-4-ene-3,17-dione
19-Oxoandrost-4-ene-3,17-dione
7α-Hydroxy-androstenedione
3-Oxo-13,17-secoandrost-17,13α-lactone
7α-Hydroxy-testosterone
Testosterone
19-Oxotestosterone
19-Hydroxy-testosterone
17β-Estradiol
Estriol
16-Glucuronide-estriol
2-Methoxy-estradiol-17β-3-glucuronide
2-Methoxy-estradiol-17β
2-Methoxy-estradiol-17β-3-sulfate
2-Hydroxy-estradiol-17β
6β-Hydroxy-estradiol-17β
C
C19-Steroids
C18-Steroids
5α-Dihydro-testosterone
Androstan-3alpha,17beta-diol
5β-Dihydro-testosterone

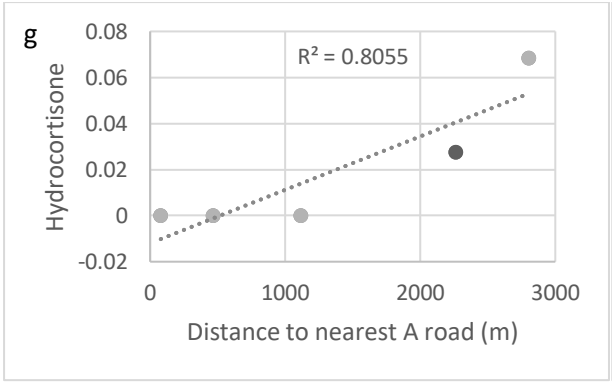
Appendix 4.7 Distance to nearest road correlated against the concentration (µM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

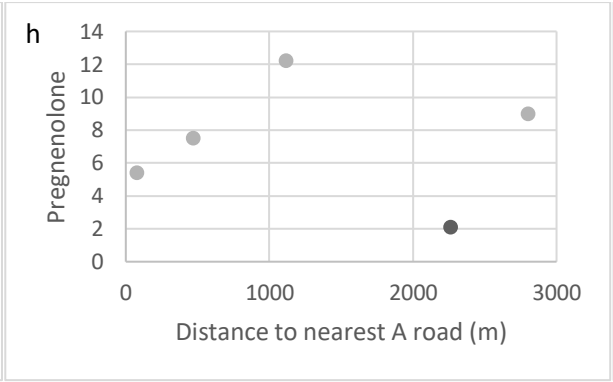


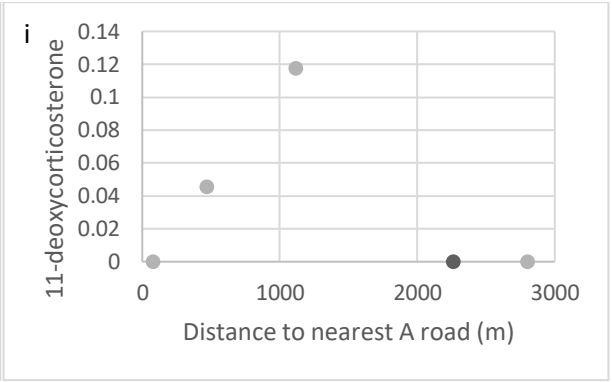

g
Hydrocortisone
0.08
0.06
0.04
0.02
0
0
100
200
300
400
500
Distance to all (m)
h
Pregnenolone
14
12
10
8
6
4
2
0
0
100
200
300
400
500
Distance to nearest road (m)
i
11-deoxycorticosterone
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
100
200
300
400
500
Distance to nearest road (m)
j
17α-Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
0
100
200
300
400
500
Distance to nearest road (m)
k
17β-Estradiol
6
5
4
3
2
1
0
0
100
200
300
400
500
Distance to nearest road (m)

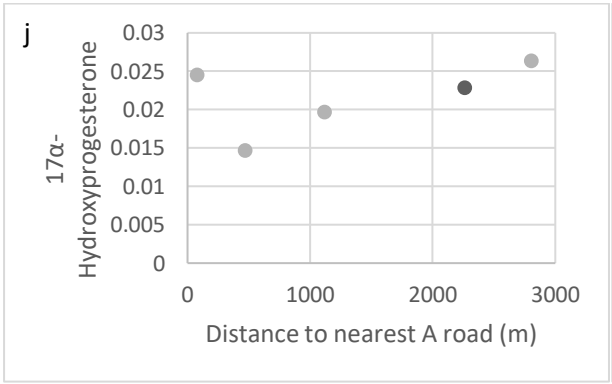
Appendix 4.8 Distance to nearest A road correlated against the concentration (µM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

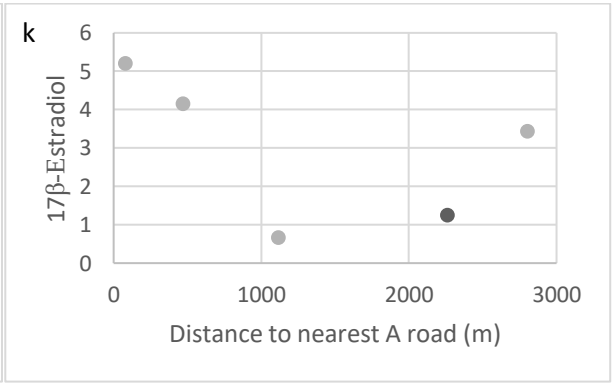



g
Hydrocortisone
R² = 0.8055
0.08
0.06
0.04
0.02
0
-0.02
0
1000
2000
3000
Distance to nearest A road (m)

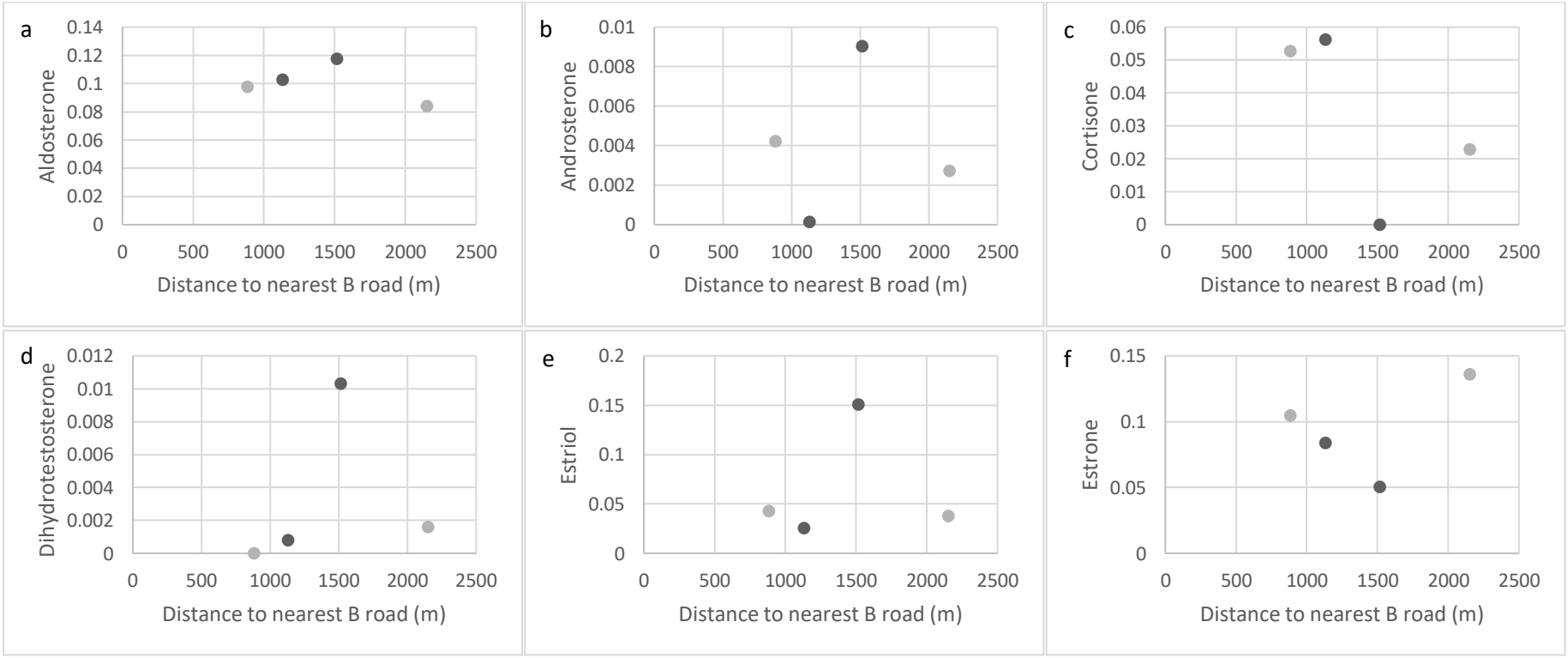

h
Pregnenolone
14
12
10
8
6
4
2
0
0
1000
2000
3000
Distance to nearest A road (m)

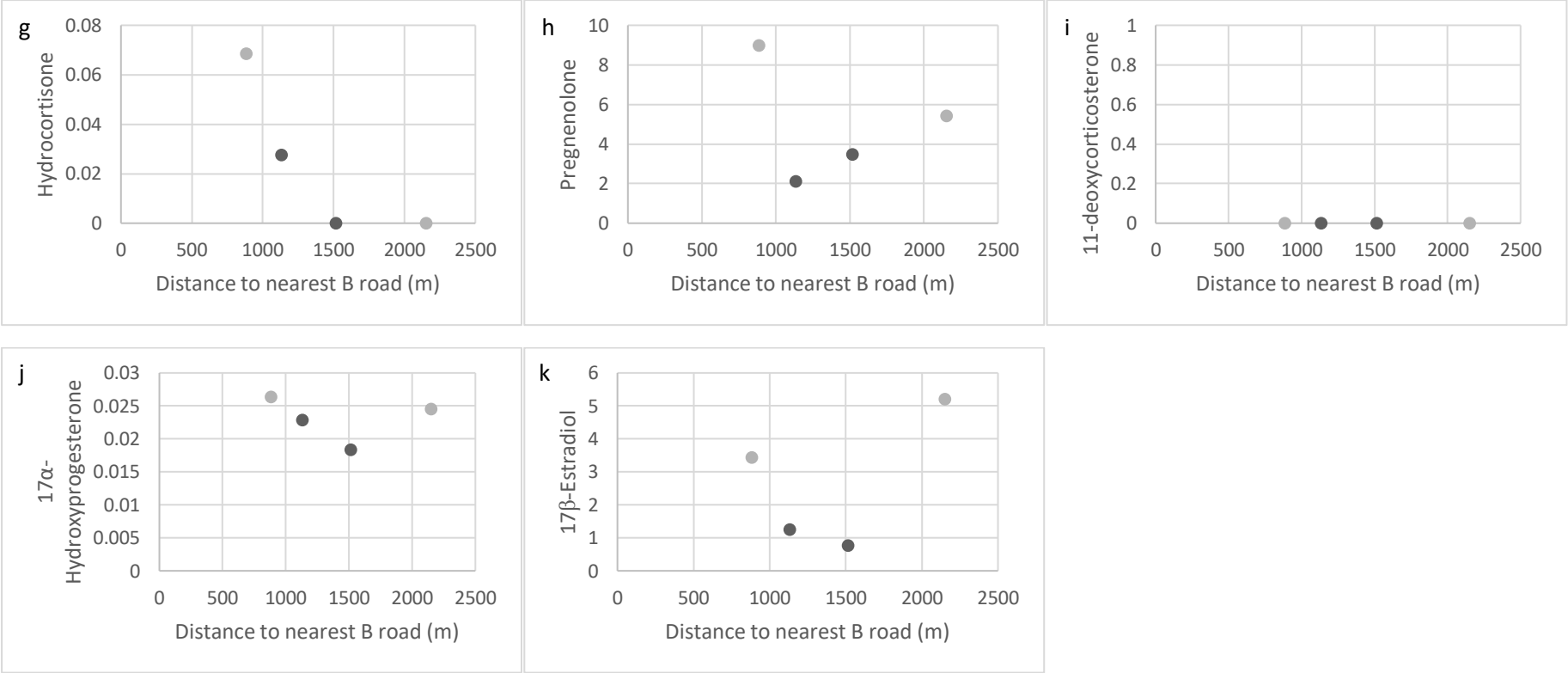

i
11-deoxycorticosterone
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
1000
2000
3000
Distance to nearest A road (m)


j
17α-Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
0
1000
2000
3000
Distance to nearest A road (m)

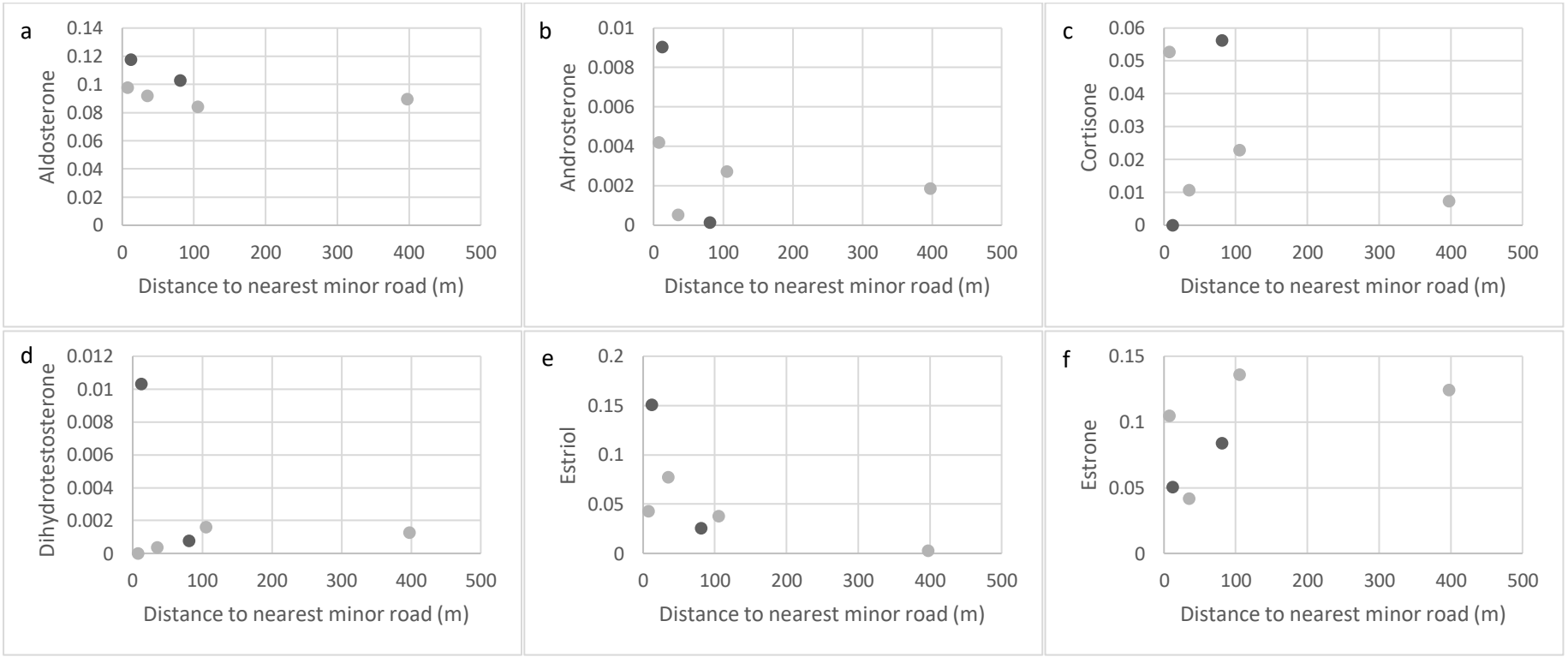

k
17β-Estradiol
6
5
4
3
2
1
0
0
1000
2000
3000
Distance to nearest A road (m)

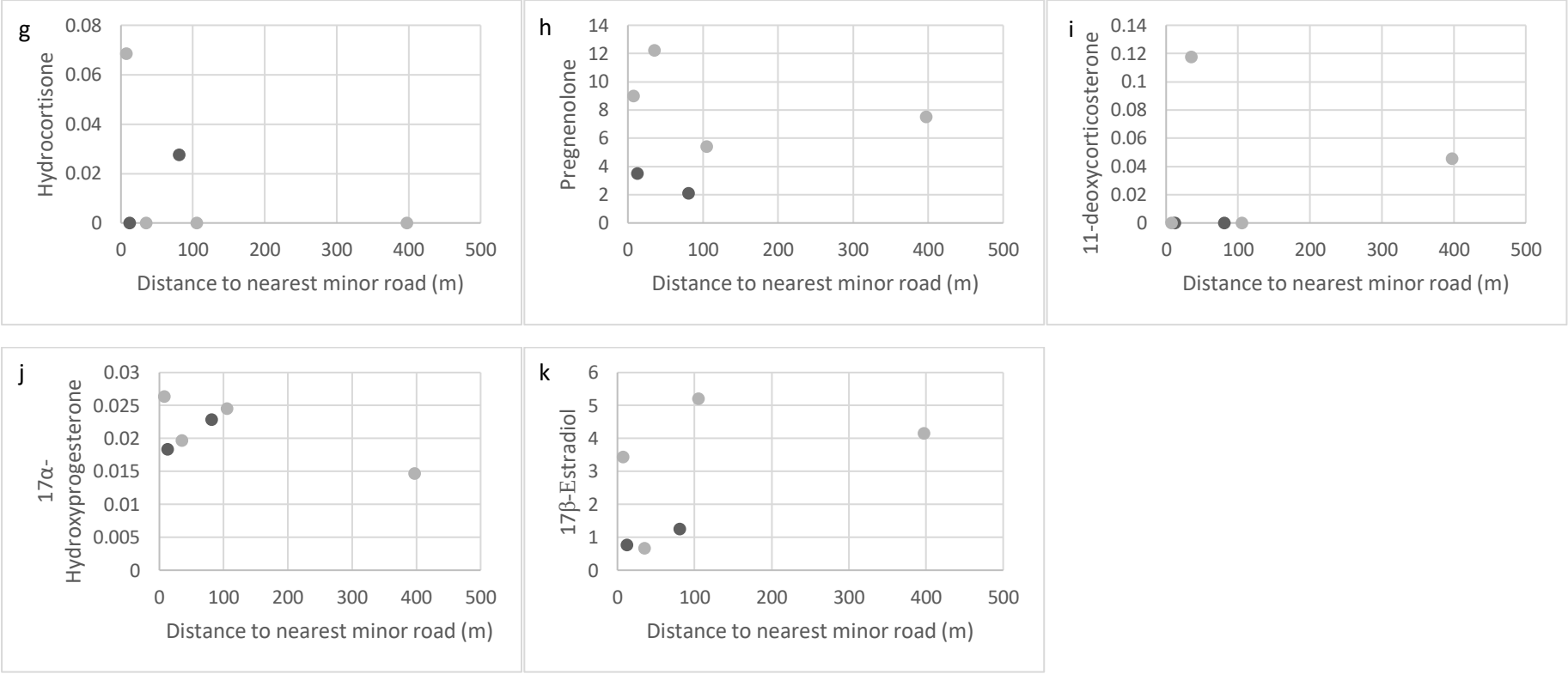
Appendix 4.9 Distance to nearest B road correlated against the concentration (µM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).



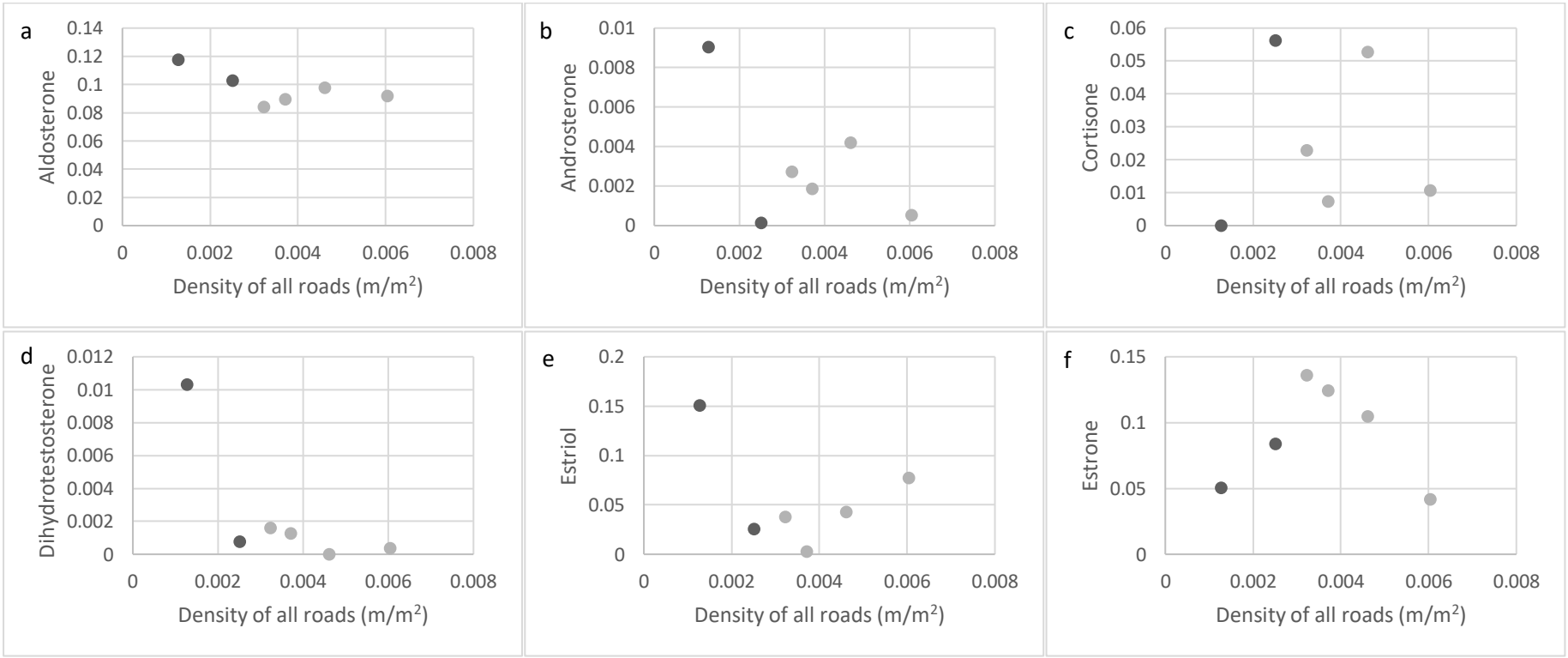

g
Hydrocortisone
0.08
0.06
0.04
0.02
0
0 500 1000 1500 2000 2500
Distance to nearest B road (m)
h
Pregnenolone
10
8
6
4
2
0
0 500 1000 1500 2000 2500
Distance to nearest B road (m)
i
11-deoxycorticosterone
1
0.8
0.6
0.4
0.2
0
0 500 1000 1500 2000 2500
Distance to nearest B road (m)
j
17α-Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
0 500 1000 1500 2000 2500
Distance to nearest B road (m)
k
17β-Estradiol
6
5
4
3
2
1
0
0 500 1000 1500 2000 2500
Distance to nearest B road (m)

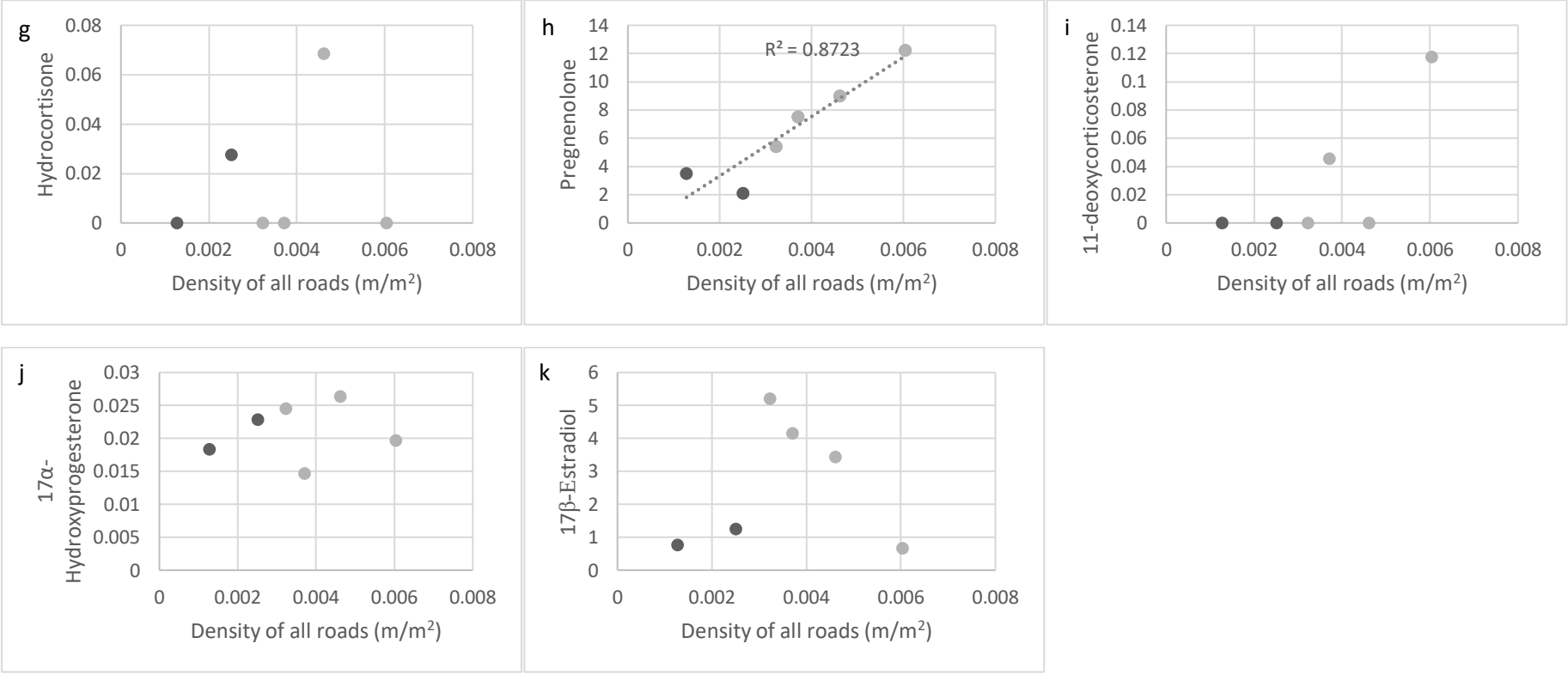
Appendix 4.10 Distance to minor road correlated against the concentration (µM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).



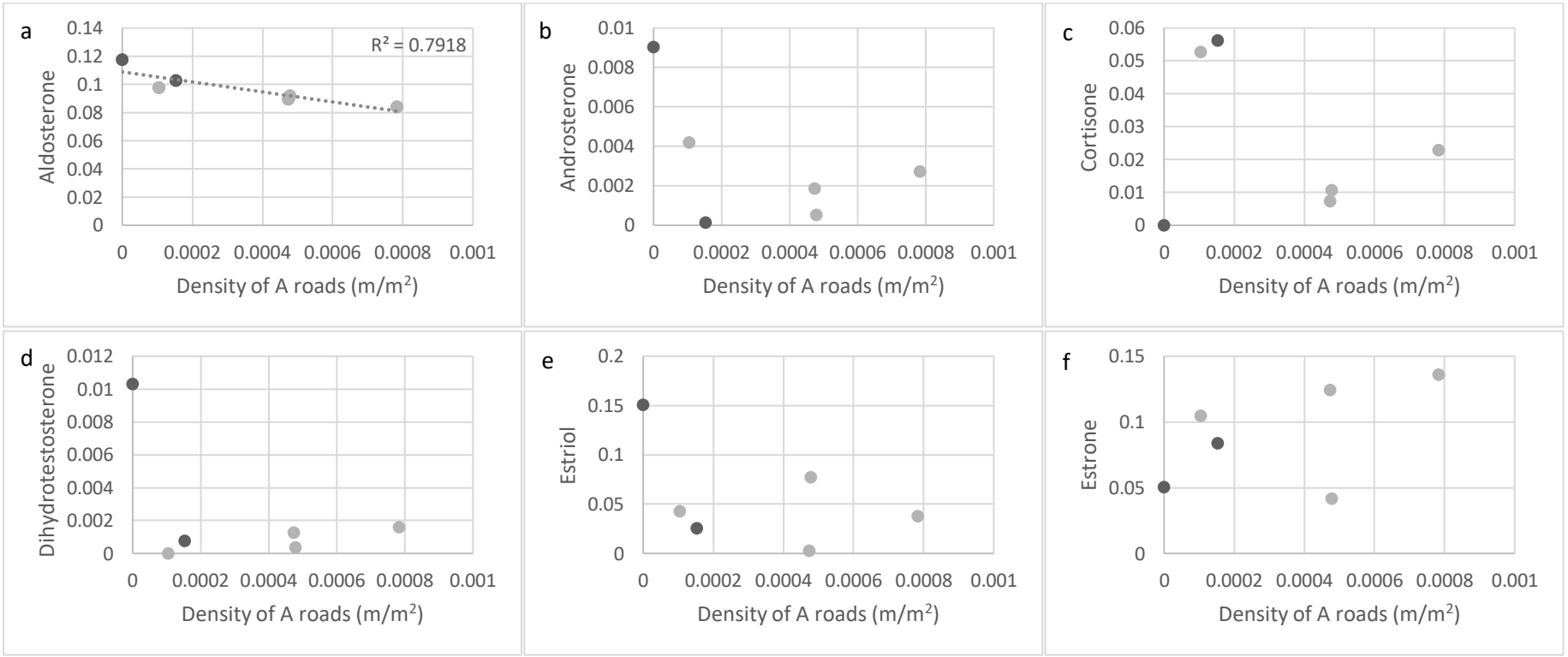

g
Hydrocortisone
0.08
0.06
0.04
0.02
0
0
100
200
300
400
500
Distance to nearest minor road (m)
h
Pregnenolone
14
12
10
8
6
4
2
0
0
100
200
300
400
500
Distance to nearest minor road (m)
i
11-deoxycorticosterone
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
100
200
300
400
500
Distance to nearest minor road (m)
j
17α-Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
0
100
200
300
400
500
Distance to nearest minor road (m)
k
17β-Estradiol
6
5
4
3
2
1
0
0
100
200
300
400
500
Distance to nearest minor road (m)

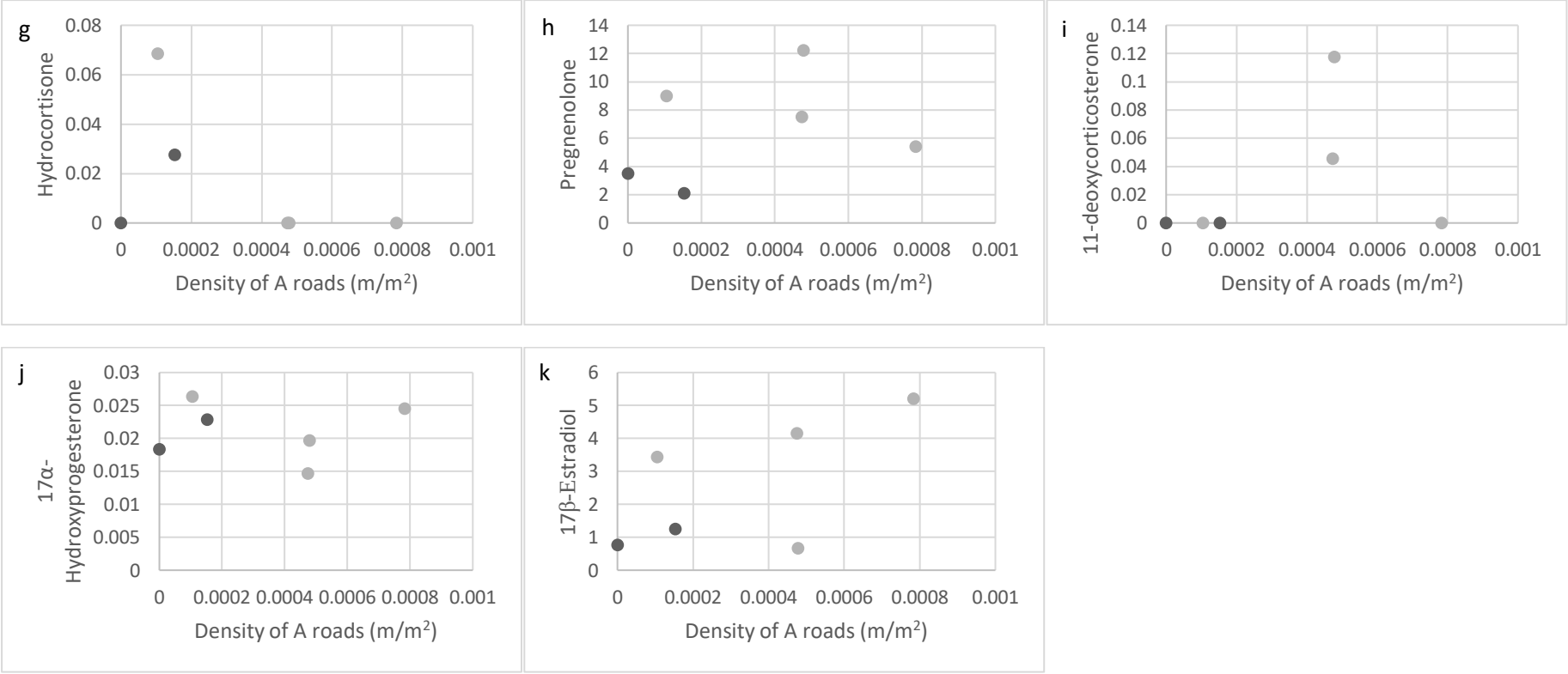
Appendix 4.11 Density of all roads correlated against the concentration (µM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).



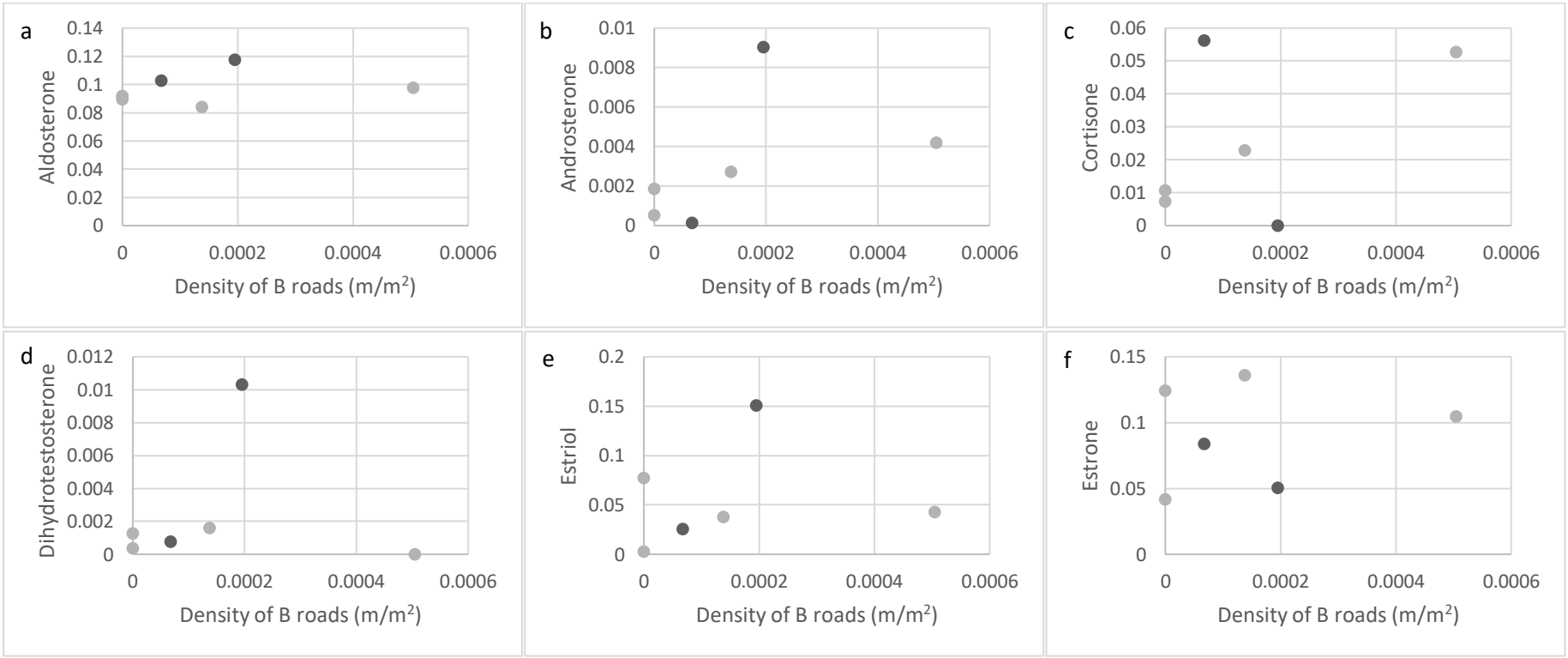

g
Hydrocortisone
0.08
0.06
0.04
0.02
0
0
0.002
0.004
0.006
0.008
Density of all roads (m/m²)
h
Pregnenolone
14
12
10
8
6
4
2
0
R² = 0.8723
0
0.002
0.004
0.006
0.008
Density of all roads (m/m²)
i
11-deoxycorticosterone
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
0.002
0.004
0.006
0.008
Density of all roads (m/m²)
j
17α-Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
0
0.002
0.004
0.006
0.008
Density of all roads (m/m²)
k
17β-Estradiol
6
5
4
3
2
1
0
0
0.002
0.004
0.006
0.008
Density of all roads (m/m²)

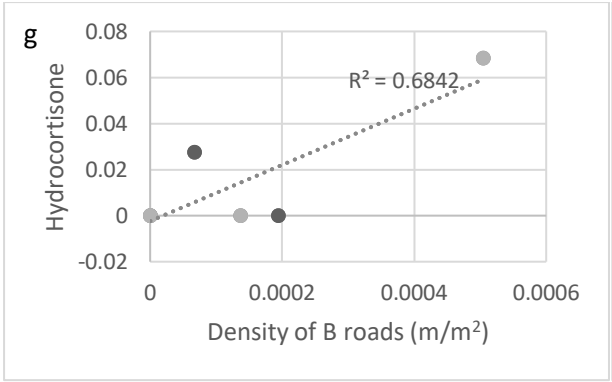
Appendix 4.12 Density of A roads correlated against the concentration (µM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

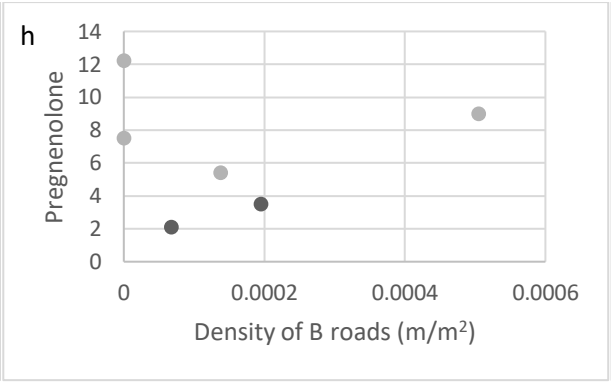


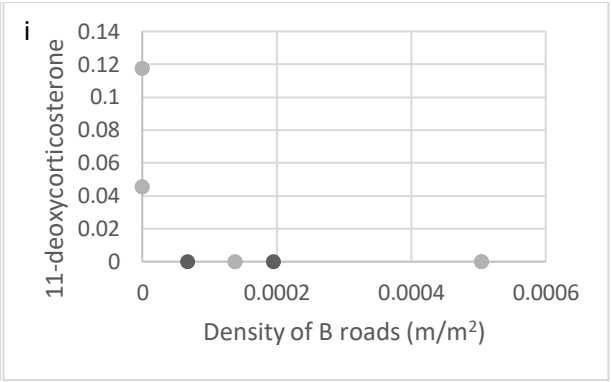

g
Hydrocortisone
0.08
0.06
0.04
0.02
0
0 0.0002 0.0004 0.0006 0.0008 0.001
Density of A roads (m/m²)
h
Pregnenolone
14
12
10
8
6
4
2
0
0 0.0002 0.0004 0.0006 0.0008 0.001
Density of A roads (m/m²)
i
11-deoxycorticosterone
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0 0.0002 0.0004 0.0006 0.0008 0.001
Density of A roads (m/m²)
j
17α-Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
0 0.0002 0.0004 0.0006 0.0008 0.001
Density of A roads (m/m²)
k
17β-Estradiol
6
5
4
3
2
1
0
0 0.0002 0.0004 0.0006 0.0008 0.001
Density of A roads (m/m²)

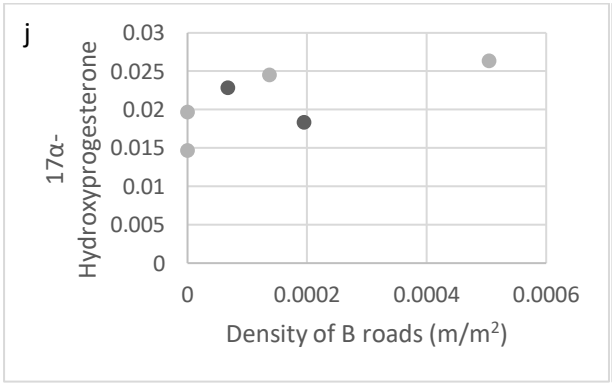
Appendix 4.13 Density of B roads correlated against the concentration (µM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

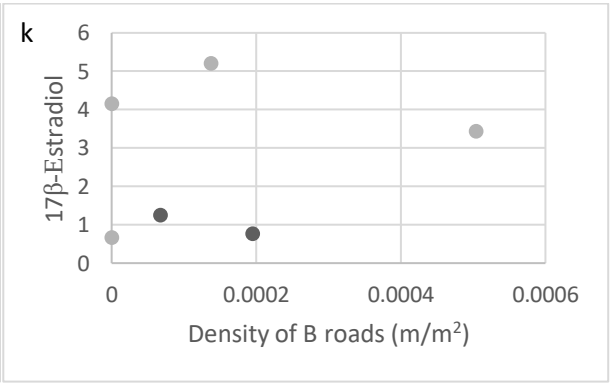



g
Hydrocortisone
0.08
0.06
0.04
0.02
0
-0.02
R² = 0.6842
0
0.0002
0.0004
0.0006
Density of B roads (m/m²)

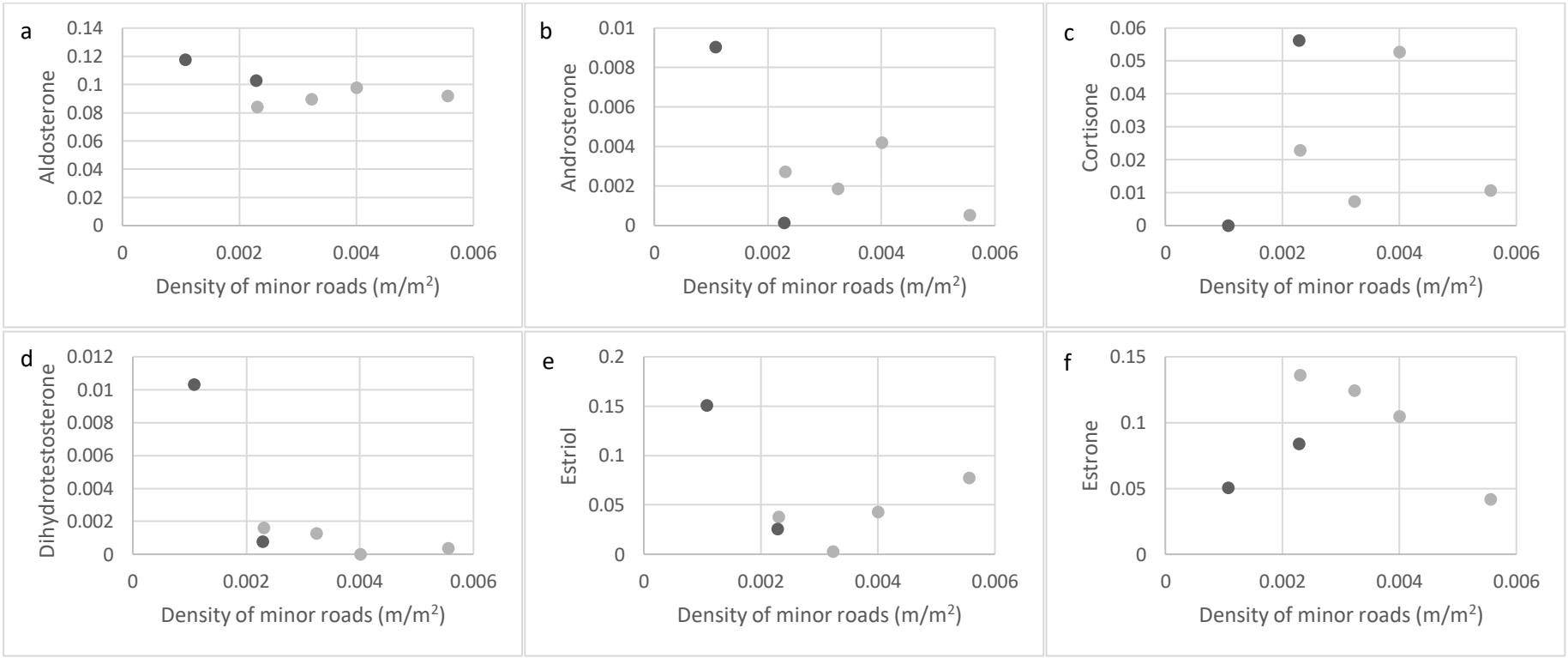

h
Pregnenolone
14
12
10
8
6
4
2
0
0
0.0002
0.0004
0.0006
Density of B roads (m/m²)

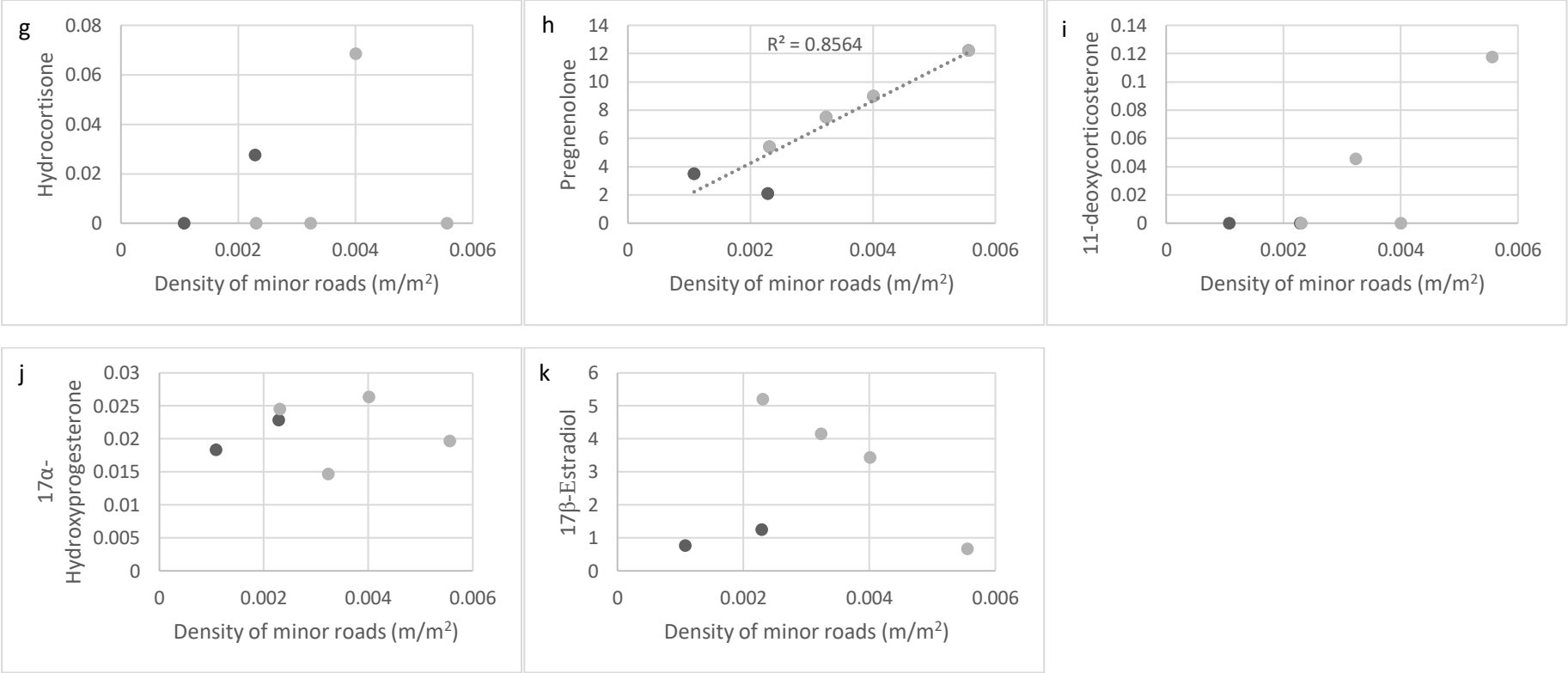

i
11-deoxycorticosterone
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
0.0002
0.0004
0.0006
Density of B roads (m/m²)


j
17α-Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
0
0.0002
0.0004
0.0006
Density of B roads (m/m²)

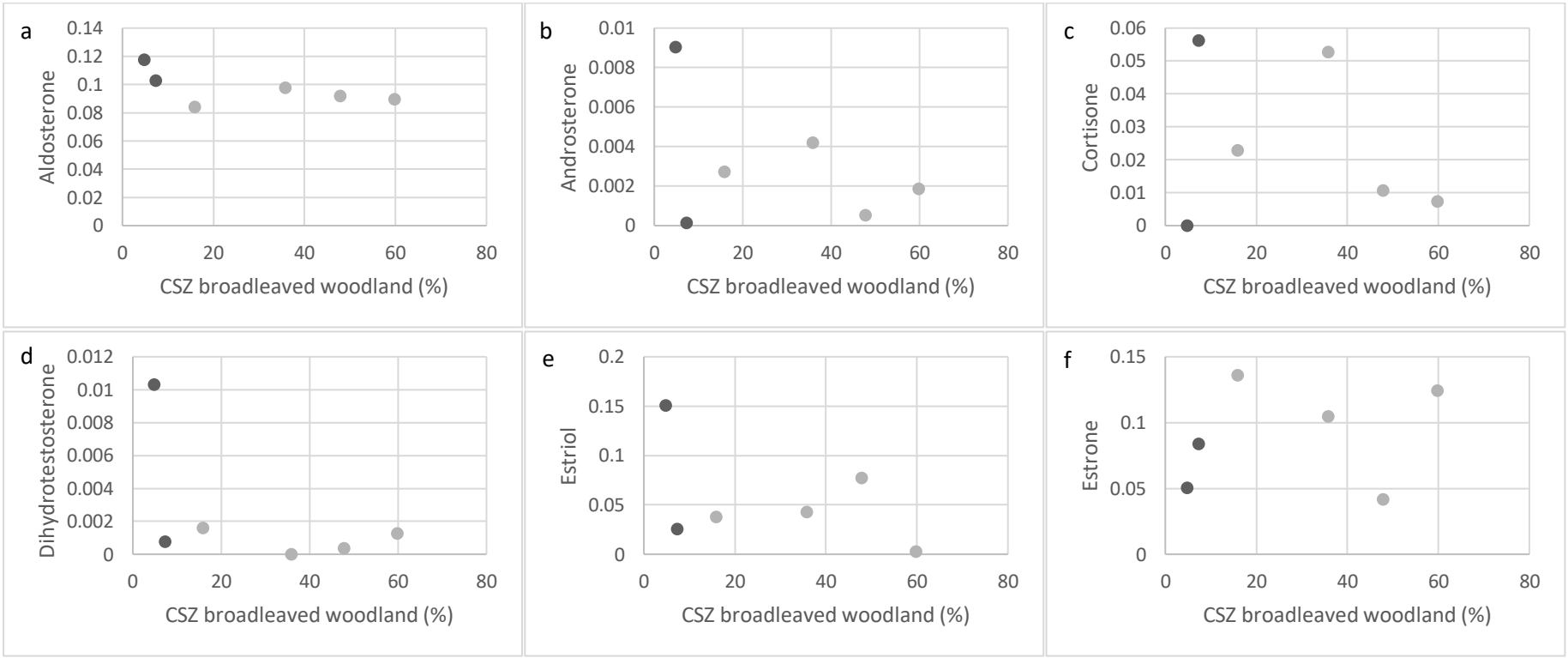

k
17β-Estradiol
6
5
4
3
2
1
0
0
0.0002
0.0004
0.0006
Density of B roads (m/m²)

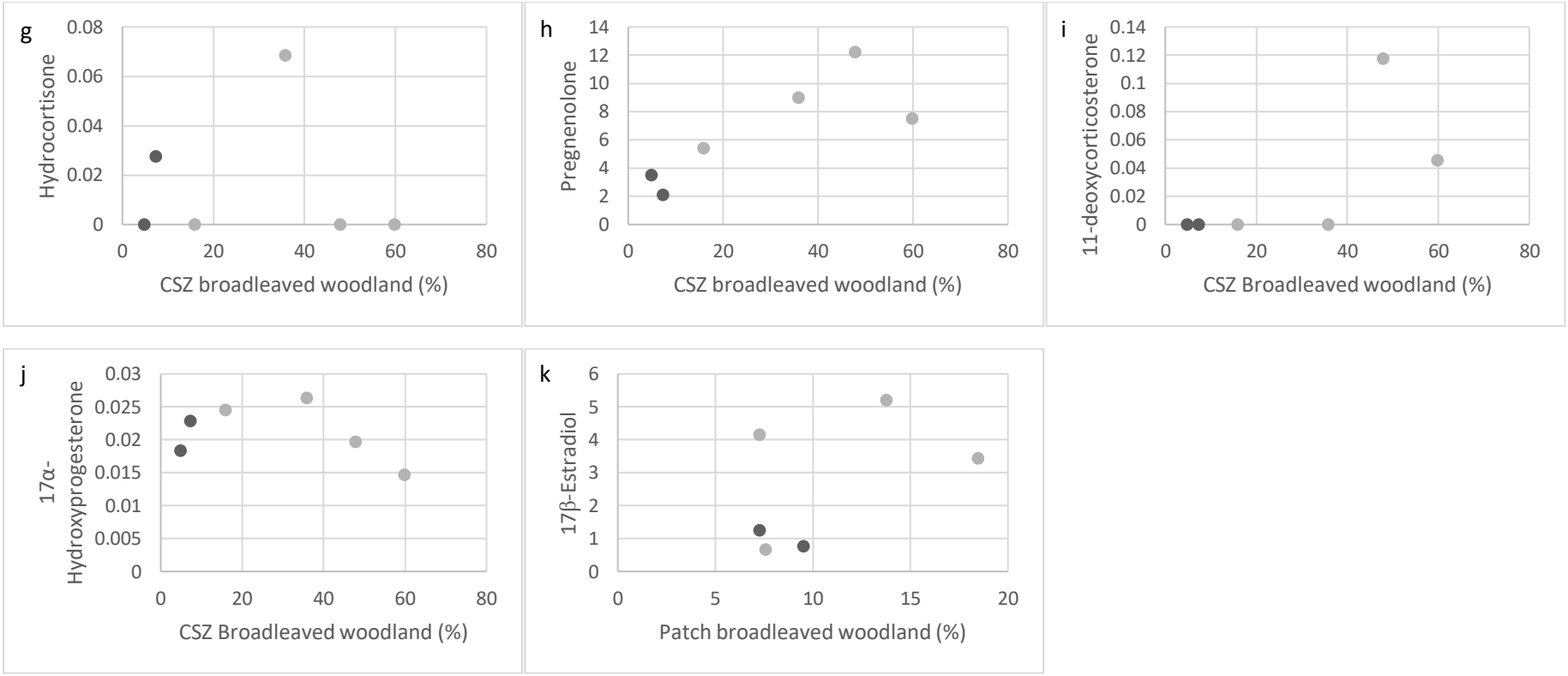
Appendix 4.14 Density of minor roads correlated against the concentration (µM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).



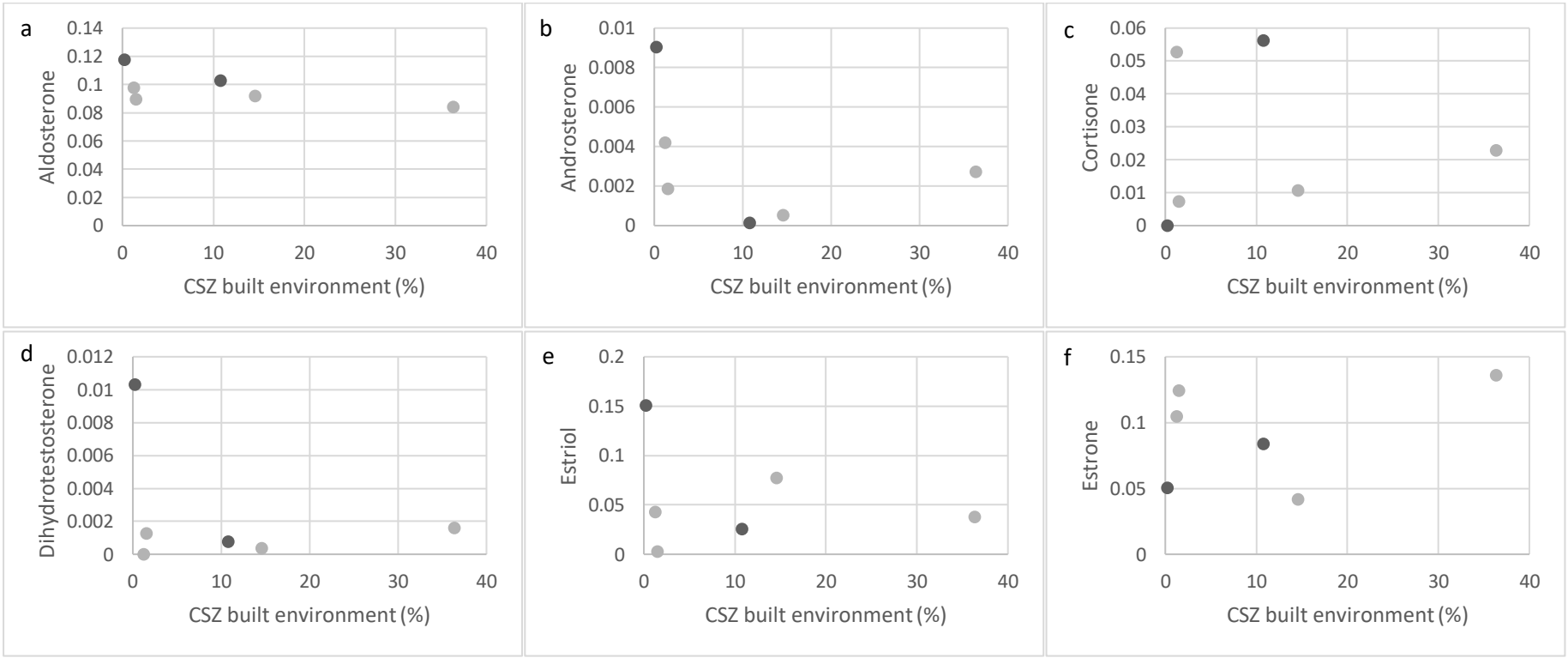

g
Hydrocortisone
0.08
0.06
0.04
0.02
0
0
0.002
0.004
0.006
Density of minor roads (m/m²)
h
Pregnenolone
R² = 0.8564
14
12
10
8
6
4
2
0
0
0.002
0.004
0.006
Density of minor roads (m/m²)
i
11-deoxycorticosterone
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
0.002
0.004
0.006
Density of minor roads (m/m²)
j
17α-Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
0
0.002
0.004
0.006
Density of minor roads (m/m²)
k
17β-Estradiol
6
5
4
3
2
1
0
0
0.002
0.004
0.006
Density of minor roads (m/m²)

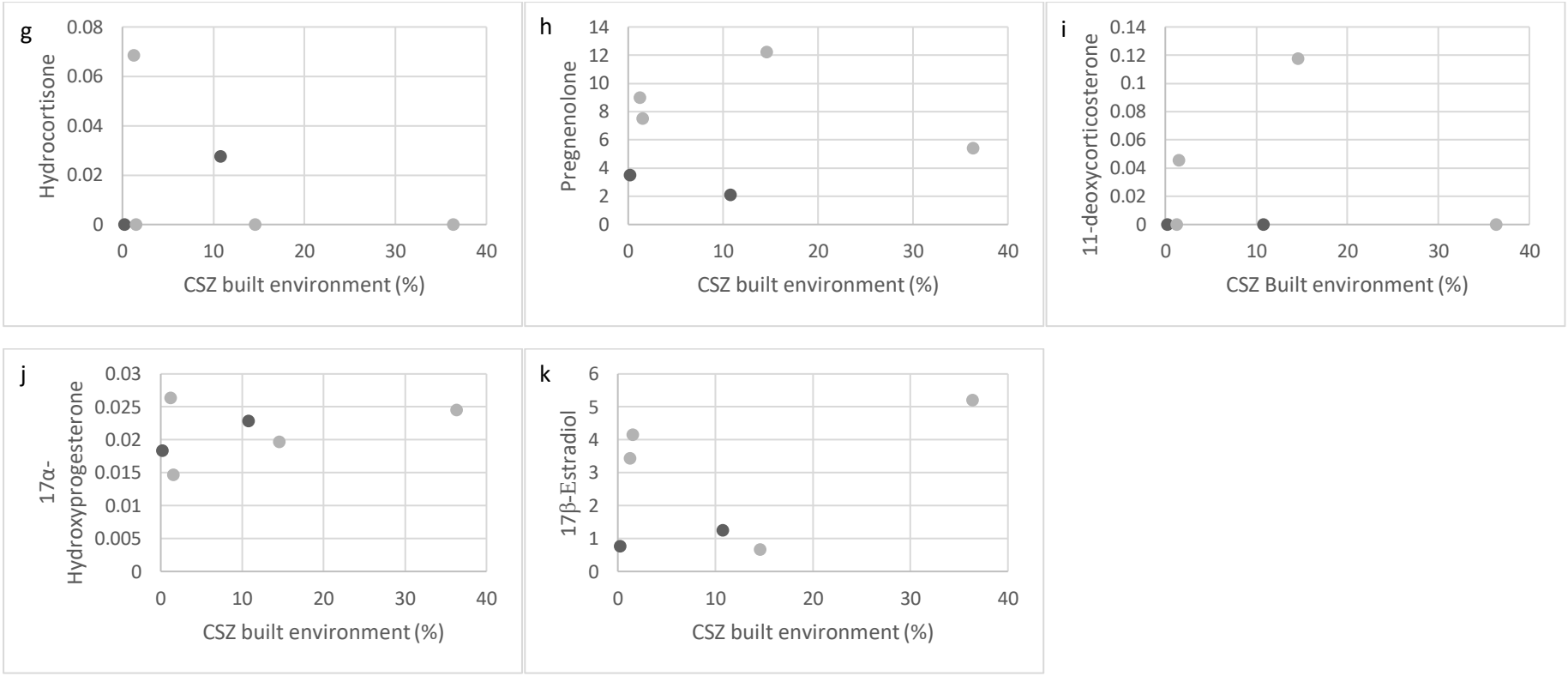
Appendix 4.15 CSZ broadleaved woodland (%) correlated against the concentration (µM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).



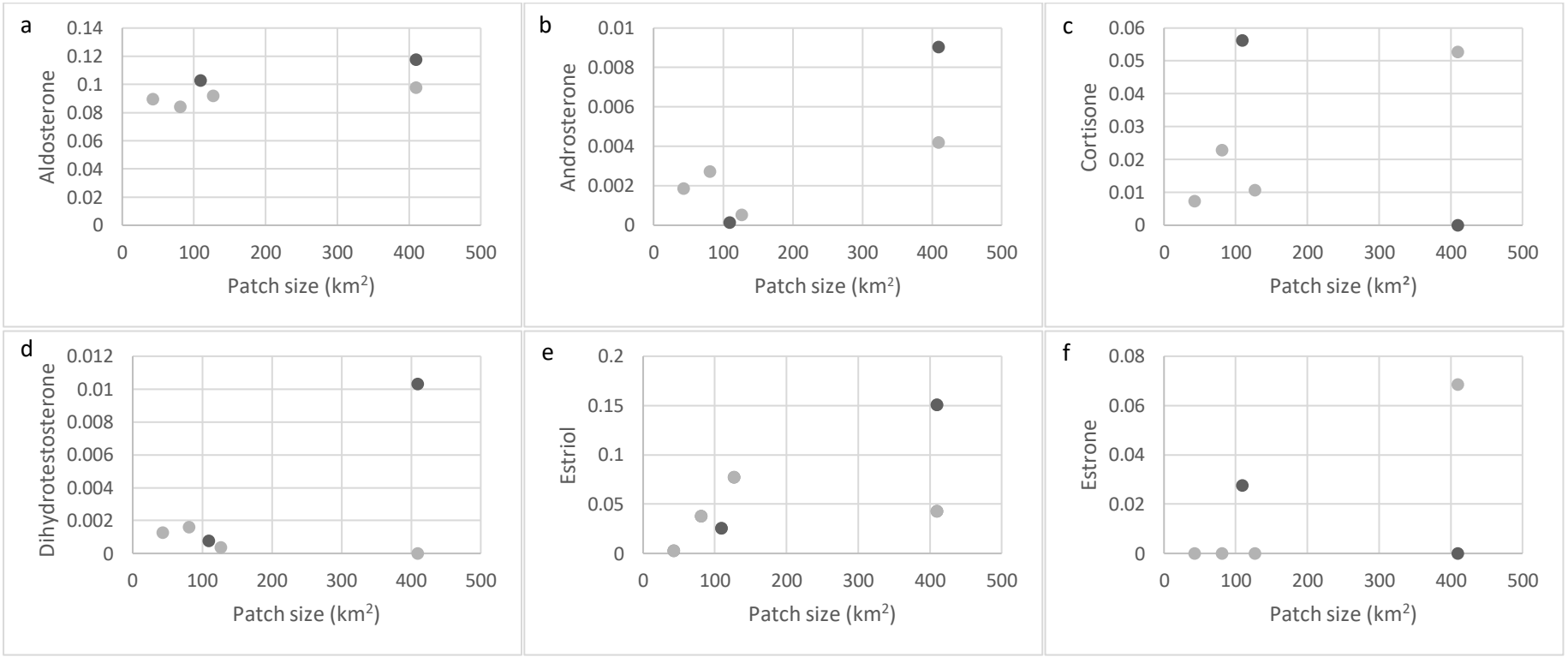

g
Hydrocortisone
0.08
0.06
0.04
0.02
0
0
20
40
60
80
CSZ broadleaved woodland (%)
h
Pregnenolone
14
12
10
8
6
4
2
0
0
20
40
60
80
CSZ broadleaved woodland (%)
i
11-deoxycorticosterone
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
20
40
60
80
CSZ Broadleaved woodland (%)
j
17α-
Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
0
20
40
60
80
CSZ Broadleaved woodland (%)
k
17β-Estradiol
6
5
4
3
2
1
0
0
5
10
15
20
Patch broadleaved woodland (%)

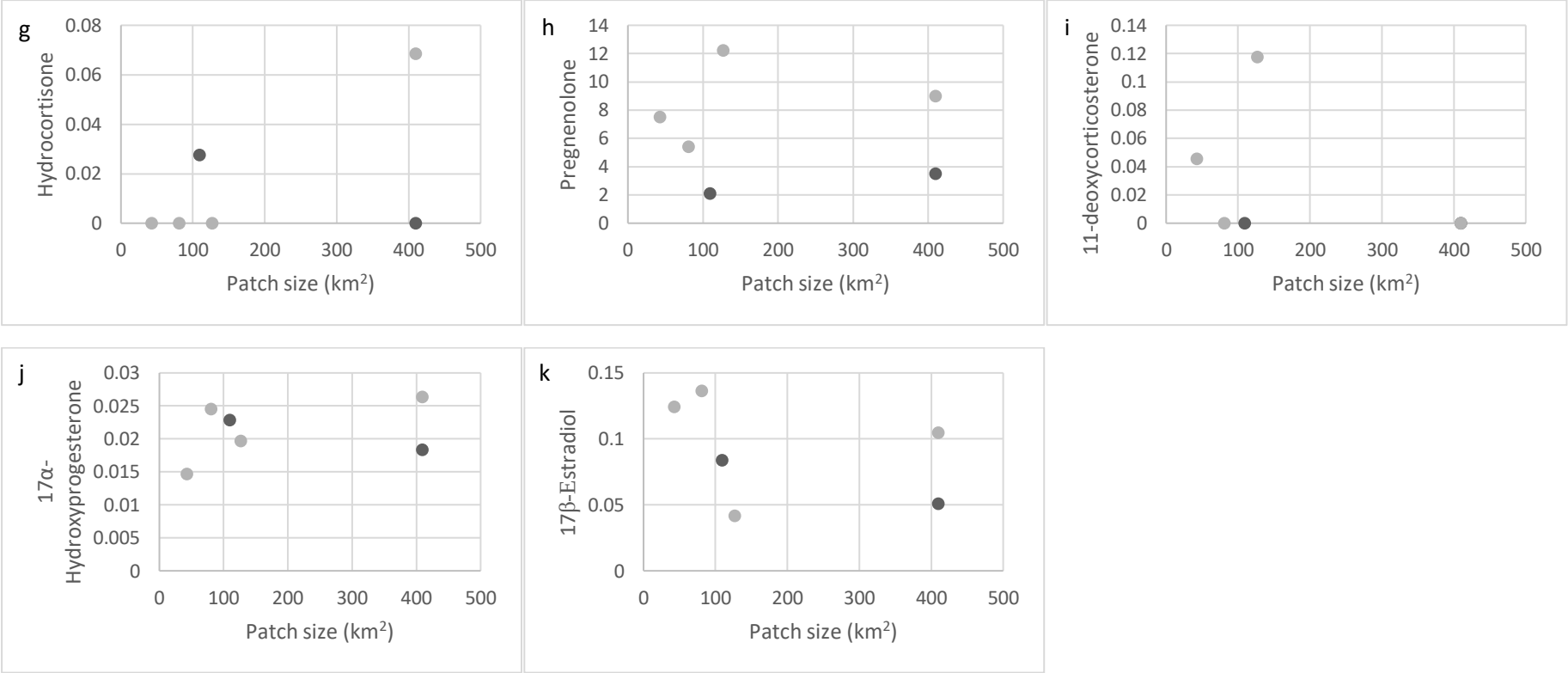
Appendix 4.16 CSZ built environment (%) correlated against the concentration (µM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).



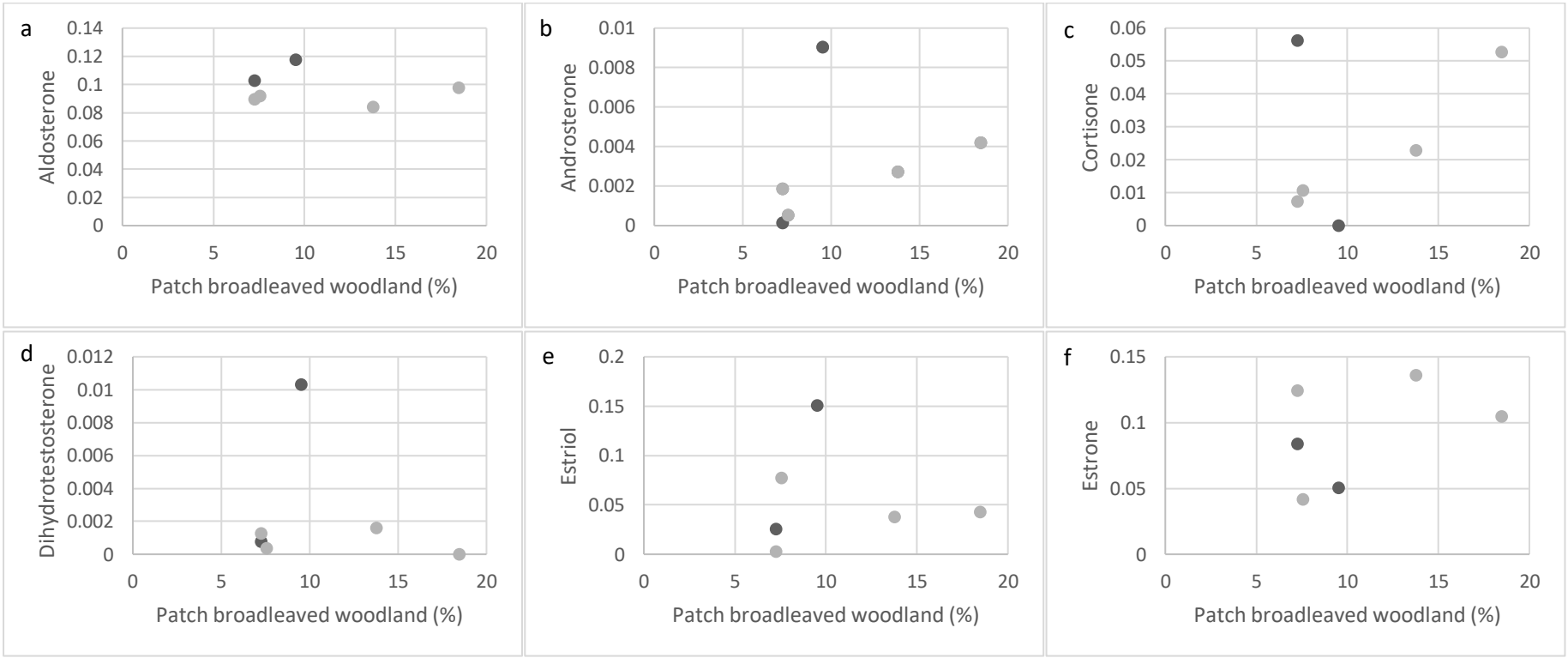

g
Hydrocortisone
0.08
0.06
0.04
0.02
0
0
10
20
30
40
CSZ built environment (%)
h
Pregnenolone
14
12
10
8
6
4
2
0
0
10
20
30
40
CSZ built environment (%)
i
11-deoxycorticosterone
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
10
20
30
40
CSZ Built environment (%)
j
17α-Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
0
10
20
30
40
CSZ built environment (%)
k
17β-Estradiol
6
5
4
3
2
1
0
0
10
20
30
40
CSZ built environment (%)

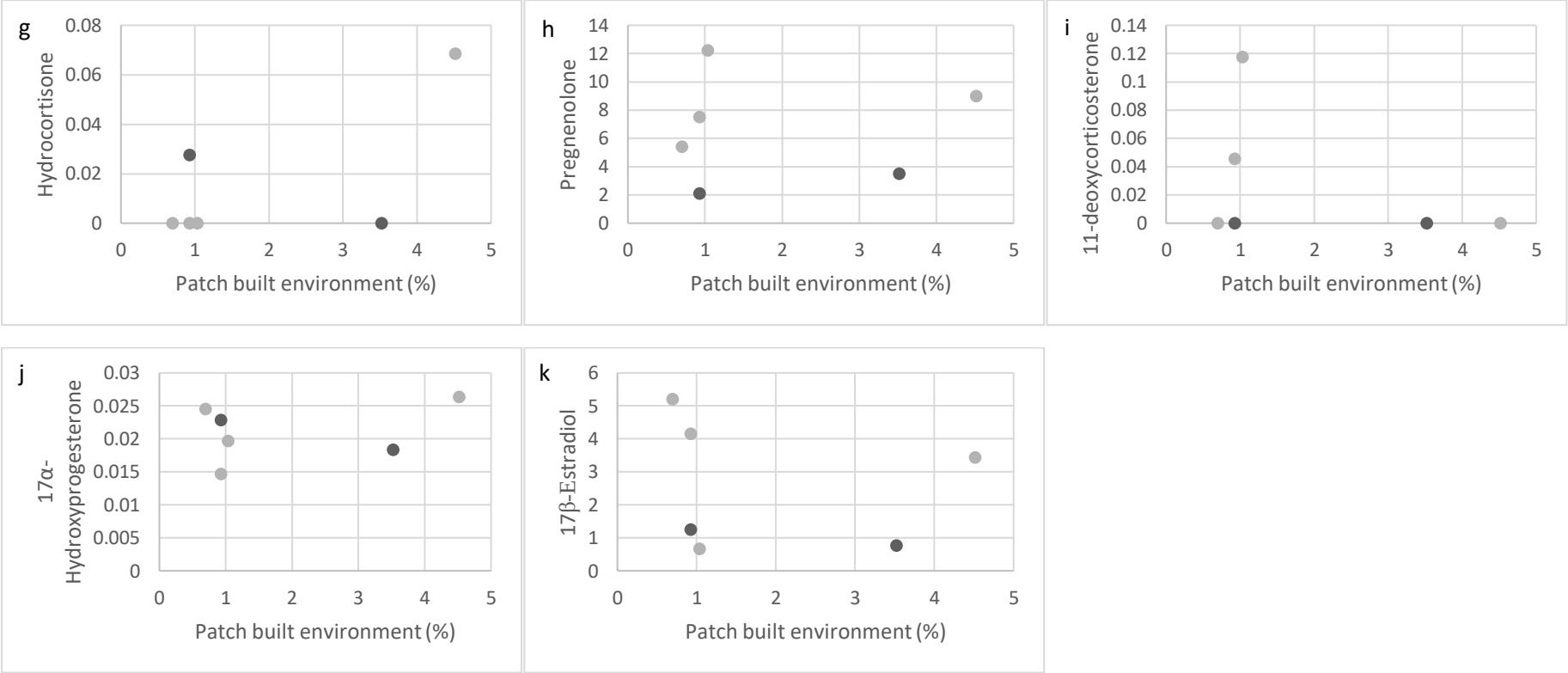
Appendix 4.17 Road-defined patch size correlated against the concentration (µM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).



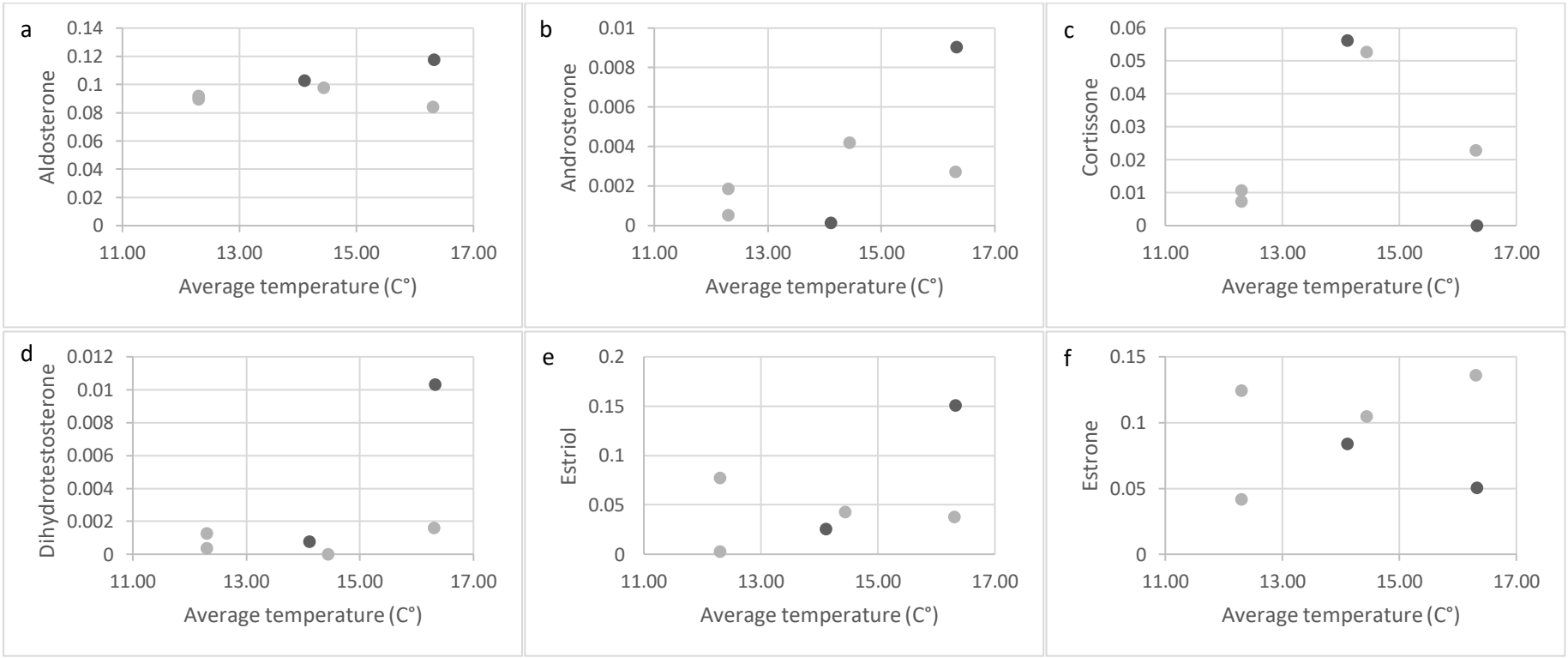

g
Hydrocortisone
0.08
0.06
0.04
0.02
0
0 100 200 300 400 500
Patch size (km²)
h
Pregnenolone
14
12
10
8
6
4
2
0
0 100 200 300 400 500
Patch size (km²)
i
11-deoxycorticosterone
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0 100 200 300 400 500
Patch size (km²)
j
17α-Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
0 100 200 300 400 500
Patch size (km²)
k
17β-Estradiol
0.15
0.1
0.05
0
0 100 200 300 400 500
Patch size (km²)

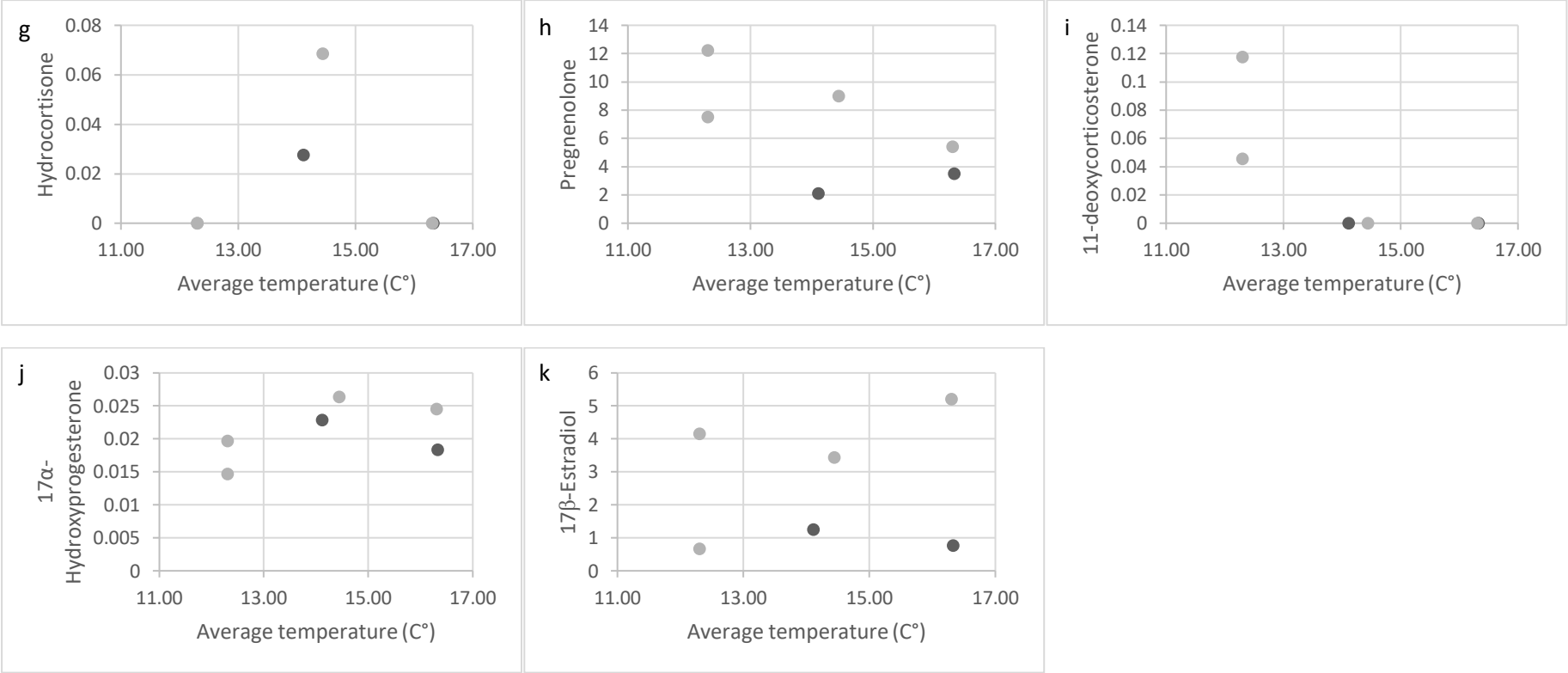
Appendix 4.18 Patch built environment (%) correlated against the concentration (μM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).



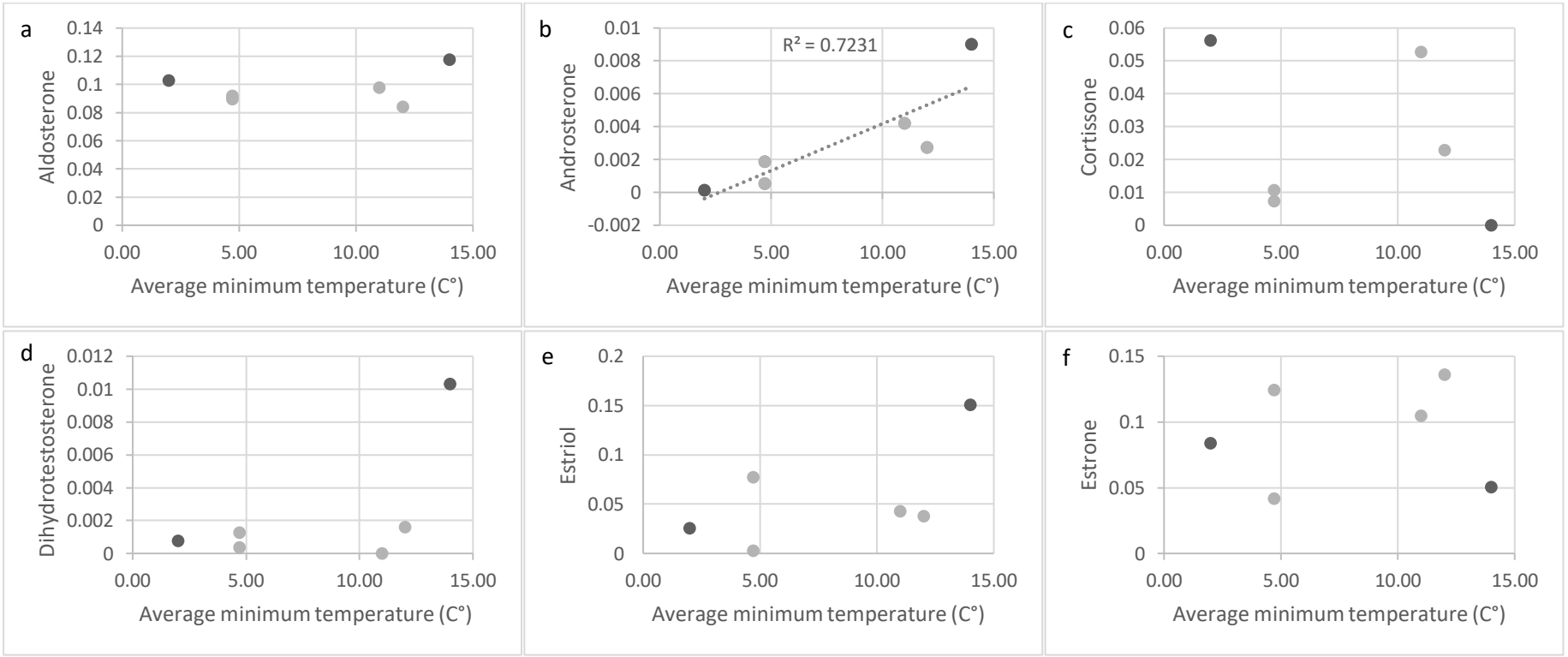

g
Hydrocortisone
0.08
0.06
0.04
0.02
0
0 1 2 3 4 5
Patch built environment (%)
h
Pregnenolone
14
12
10
8
6
4
2
0
0 1 2 3 4 5
Patch built environment (%)
i
11-deoxycorticosterone
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0 1 2 3 4 5
Patch built environment (%)
j
17α-Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
0 1 2 3 4 5
Patch built environment (%)
k
17β-Estradiol
6
5
4
3
2
1
0
0 1 2 3 4 5
Patch built environment (%)

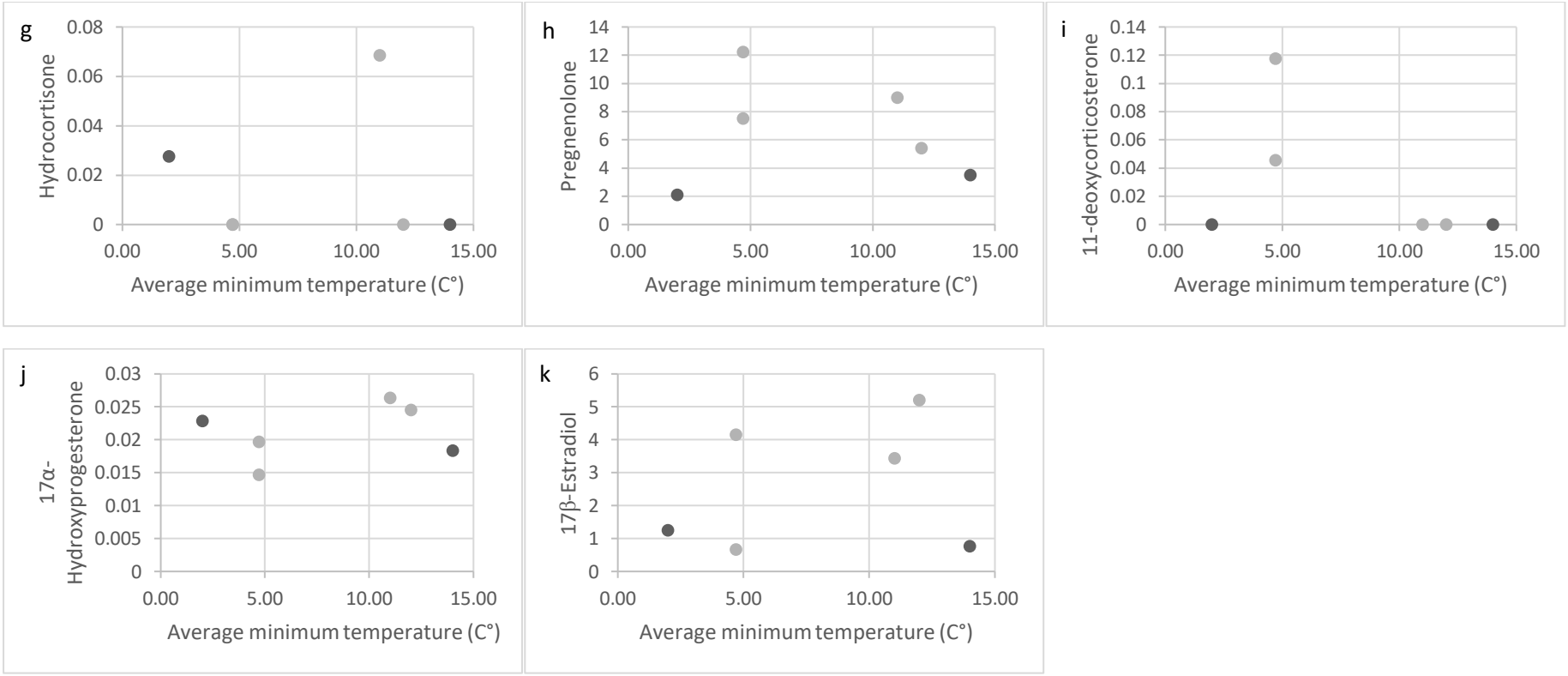
Appendix 4.19 Average temperature correlated against the concentration (µM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).



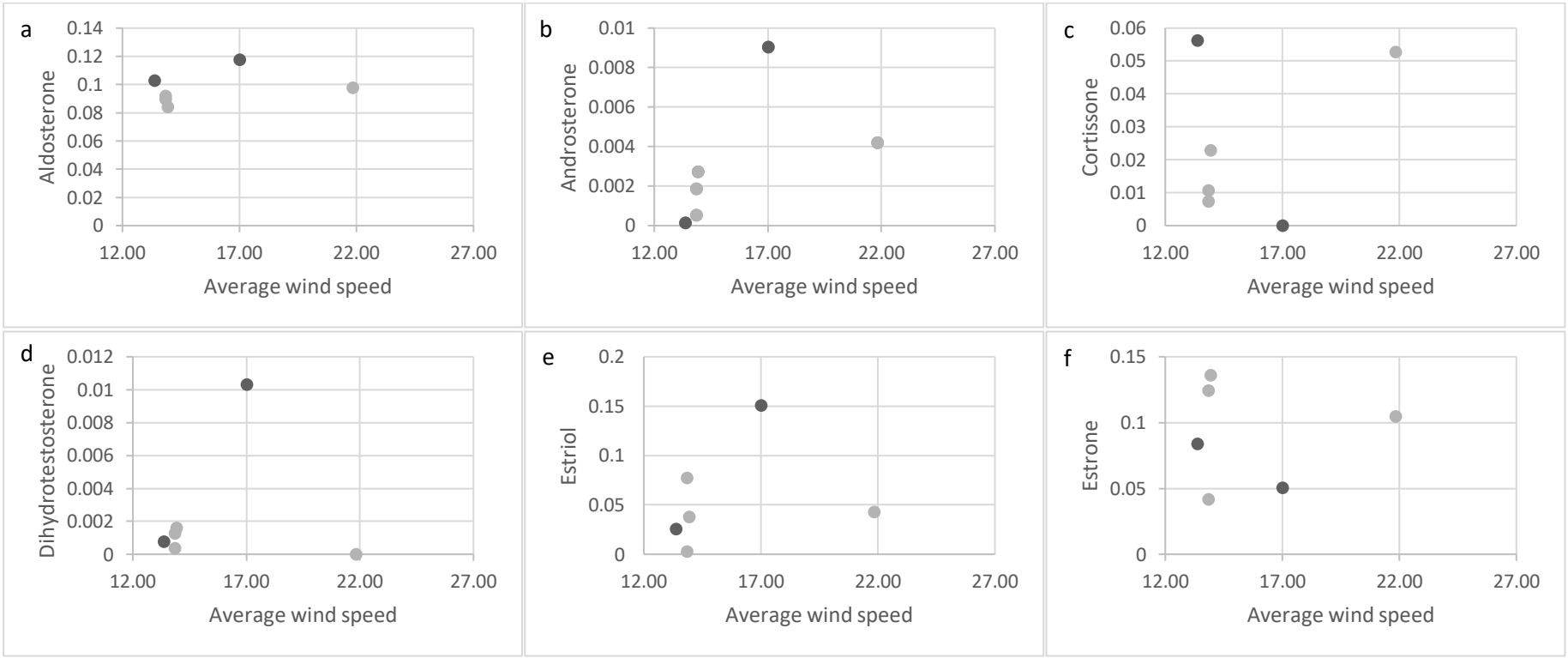

g
Hydrocortisone
0.08
0.06
0.04
0.02
0
11.00
13.00
15.00
17.00
Average temperature (C°)
h
Pregnenolone
14
12
10
8
6
4
2
0
11.00
13.00
15.00
17.00
Average temperature (C°)
i
11-deoxycorticosterone
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
11.00
13.00
15.00
17.00
Average temperature (C°)
j
17α-Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
11.00
13.00
15.00
17.00
Average temperature (C°)
k
17β-Estradiol
6
5
4
3
2
1
0
11.00
13.00
15.00
17.00
Average temperature (C°)

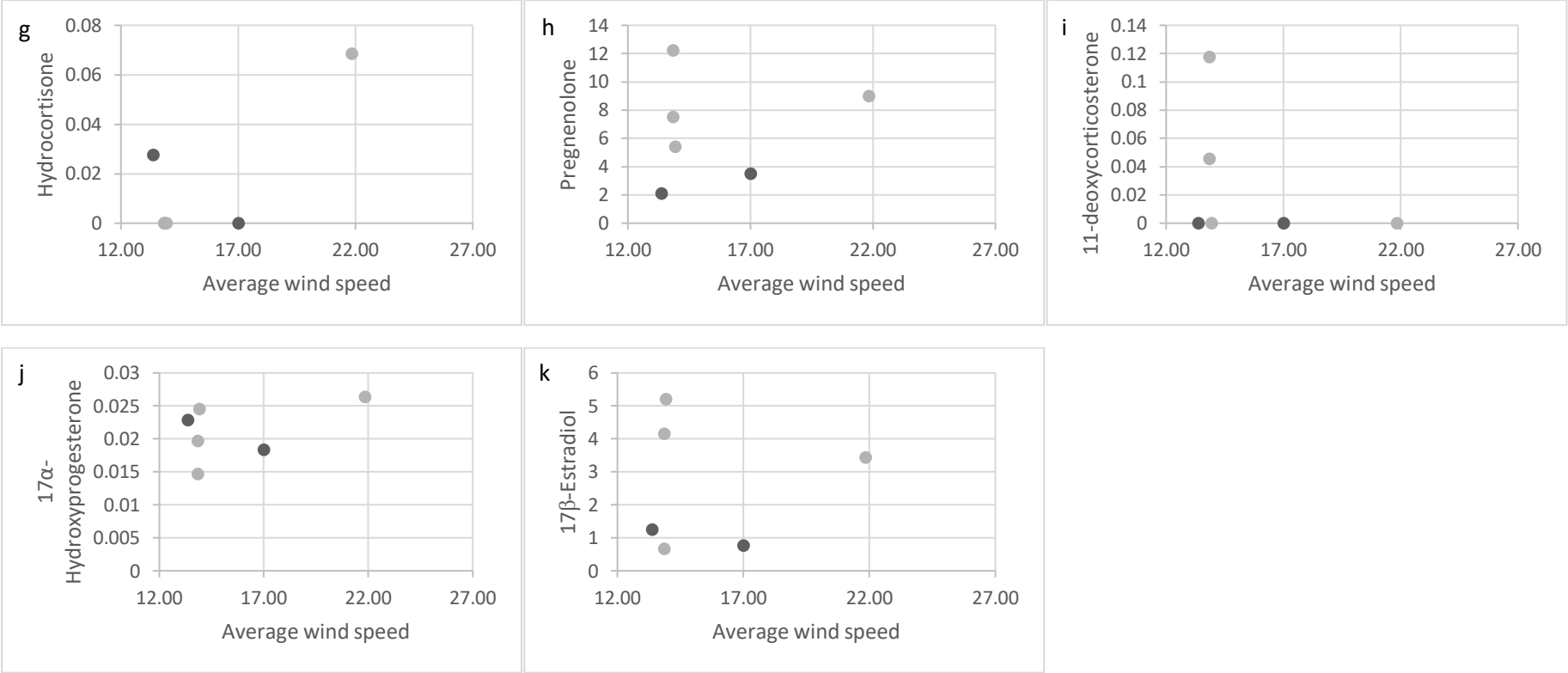
Appendix 4.20 Average minimum temperature correlated against the concentration (µM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).



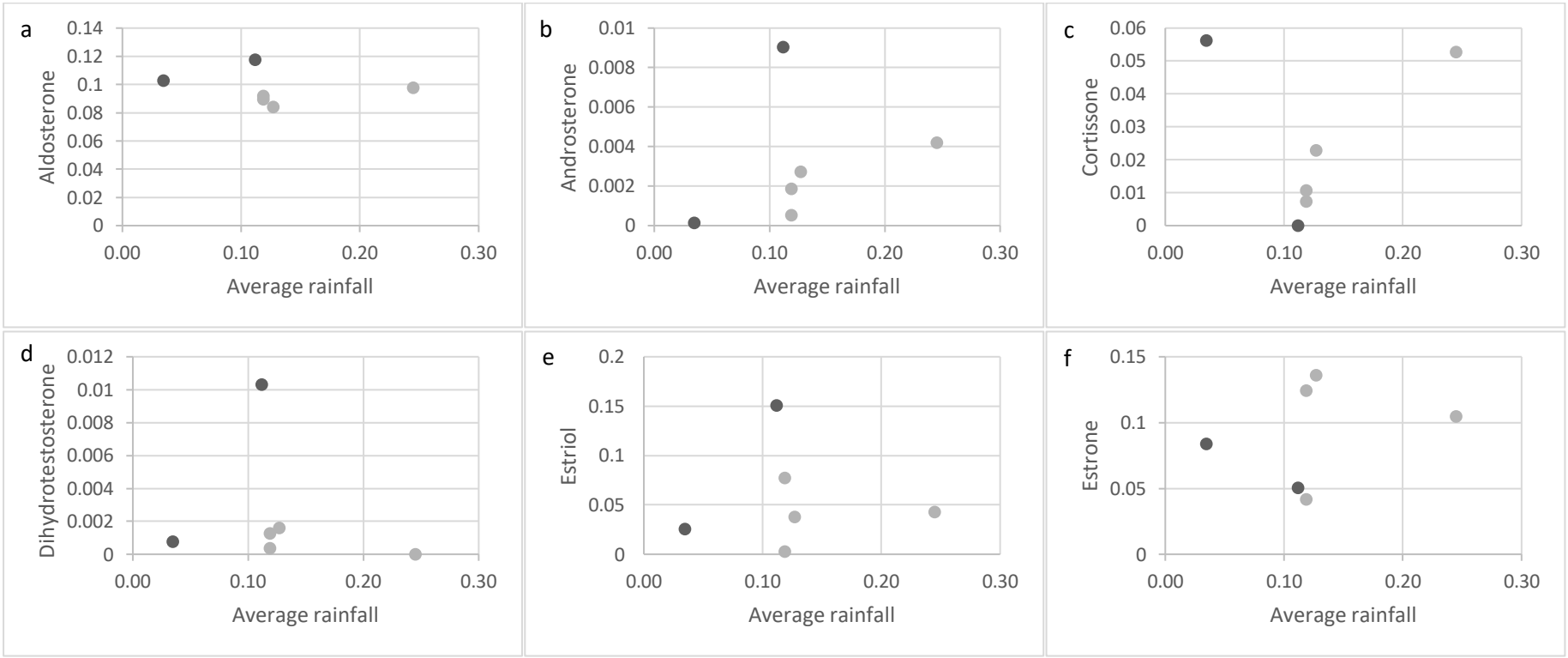

g
Hydrocortisone
0.08
0.06
0.04
0.02
0
0.00
5.00
10.00
15.00
Average minimum temperature (C°)
h
Pregnenolone
14
12
10
8
6
4
2
0
0.00
5.00
10.00
15.00
Average minimum temperature (C°)
i
11-deoxycorticosterone
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0.00
5.00
10.00
15.00
Average minimum temperature (C°)
j
17α-Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
0.00
5.00
10.00
15.00
Average minimum temperature (C°)
k
17β-Estradiol
6
5
4
3
2
1
0
0.00
5.00
10.00
15.00
Average minimum temperature (C°)

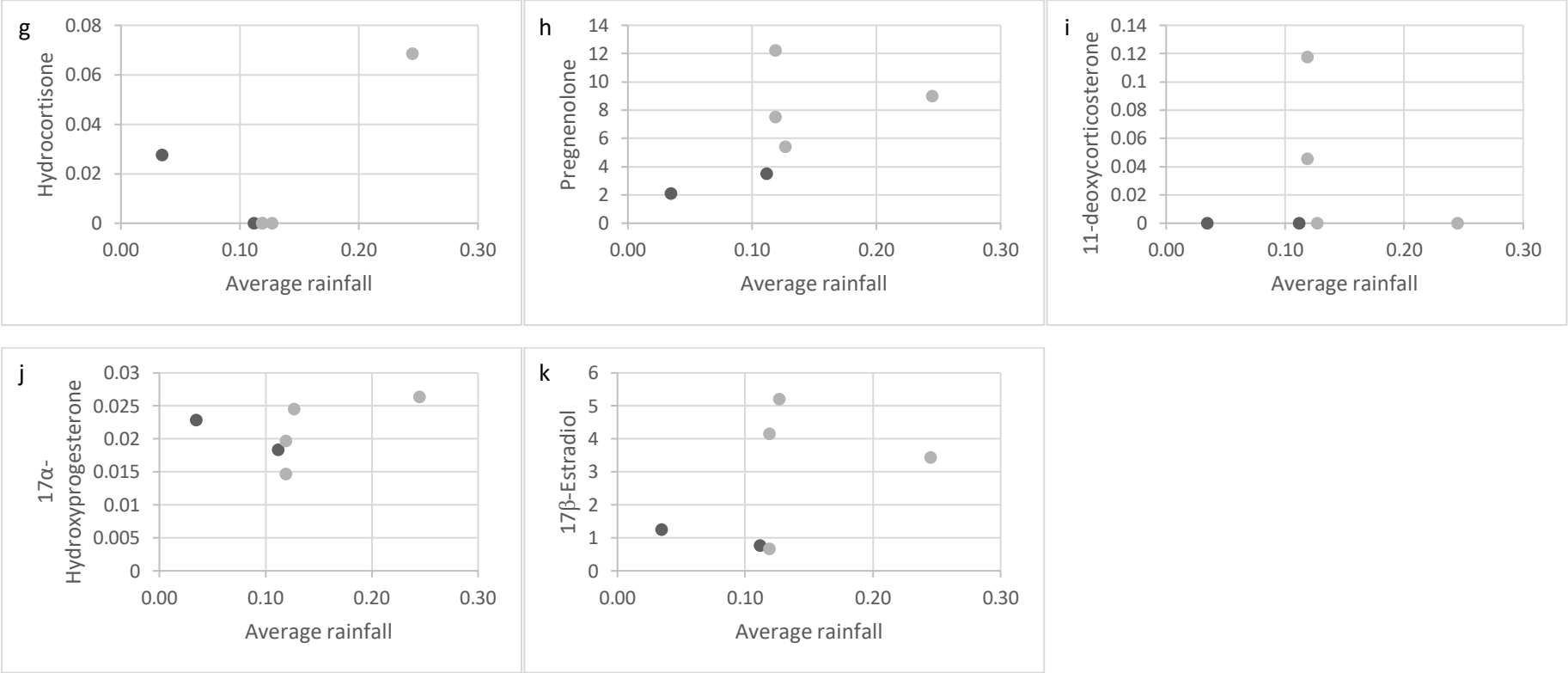
Appendix 4.21 Average wind speed correlated against the concentration (µM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).



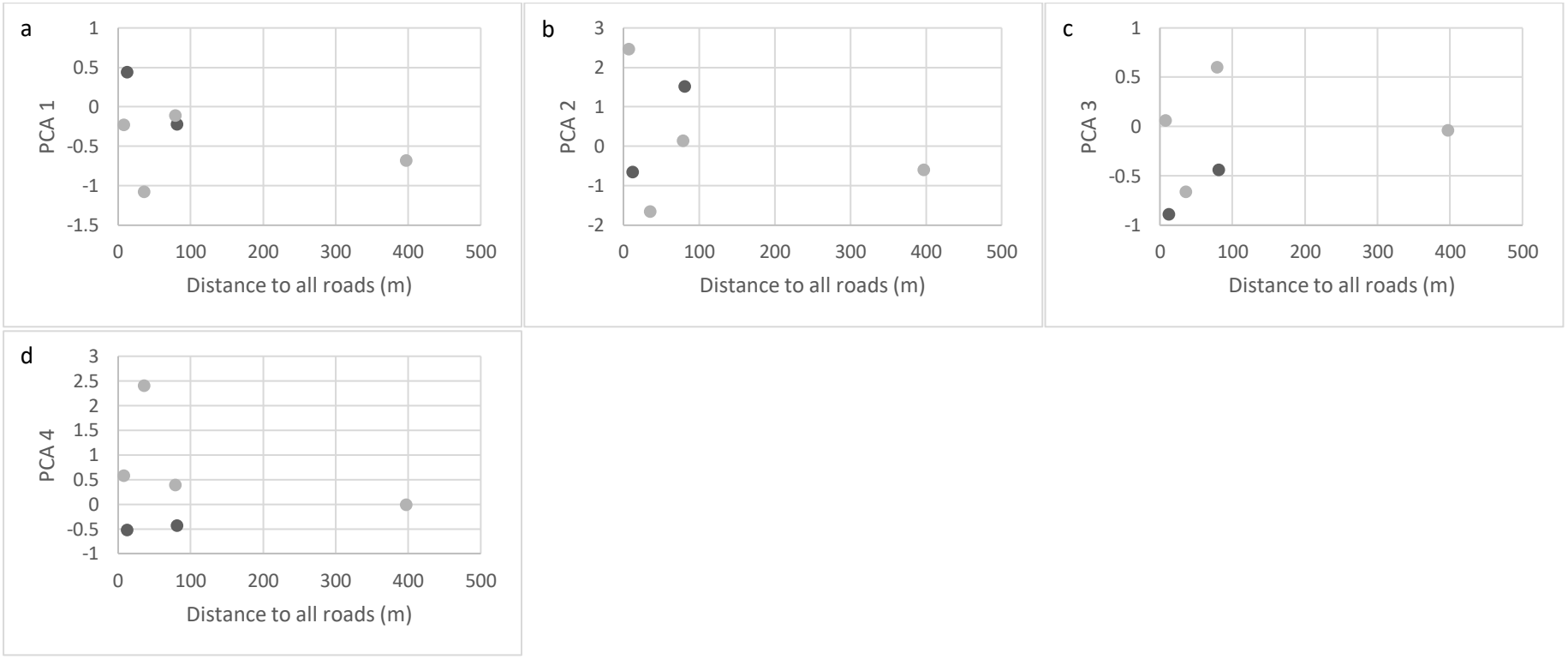

g
Hydrocortisone
0.08
0.06
0.04
0.02
0
12.00
17.00
22.00
27.00
Average wind speed
h
Pregnenolone
14
12
10
8
6
4
2
0
12.00
17.00
22.00
27.00
Average wind speed
i
11-deoxycorticosterone
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
12.00
17.00
22.00
27.00
Average wind speed
j
17α-Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
12.00
17.00
22.00
27.00
Average wind speed
k
17β-Estradiol
6
5
4
3
2
1
0
12.00
17.00
22.00
27.00
Average wind speed

Appendix 4.22 Average rainfall correlated against the concentration (μM/mg) of aldosterone (a), androsterone (b), cortisone (c), dihydrotestosterone (d), estriol (e), estrone (f), hydrocortisone (g), pregnenalone (h), 11-deoxycorticosterone (i), 17α-hydroxyprogesterone (j), 17β-estradiol (k).

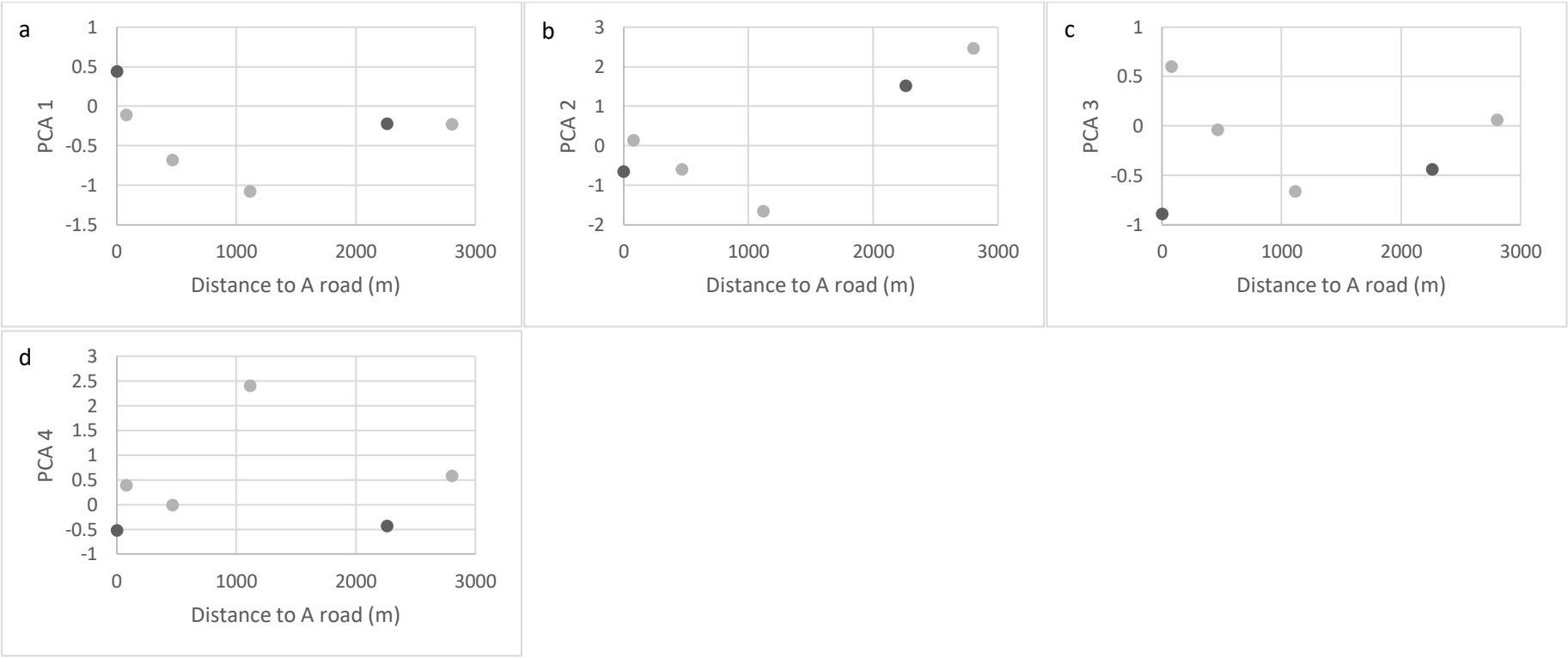



g
Hydrocortisone
0.08
0.06
0.04
0.02
0
0.00
0.10
0.20
0.30
Average rainfall
h
Pregnenolone
14
12
10
8
6
4
2
0
0.00
0.10
0.20
0.30
Average rainfall
i
11-deoxycorticosterone
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0.00
0.10
0.20
0.30
Average rainfall
j
17α-Hydroxyprogesterone
0.03
0.025
0.02
0.015
0.01
0.005
0
0.00
0.10
0.20
0.30
Average rainfall
k
17β-Estradiol
6
5
4
3
2
1
0
0.00
0.10
0.20
0.30
Average rainfall

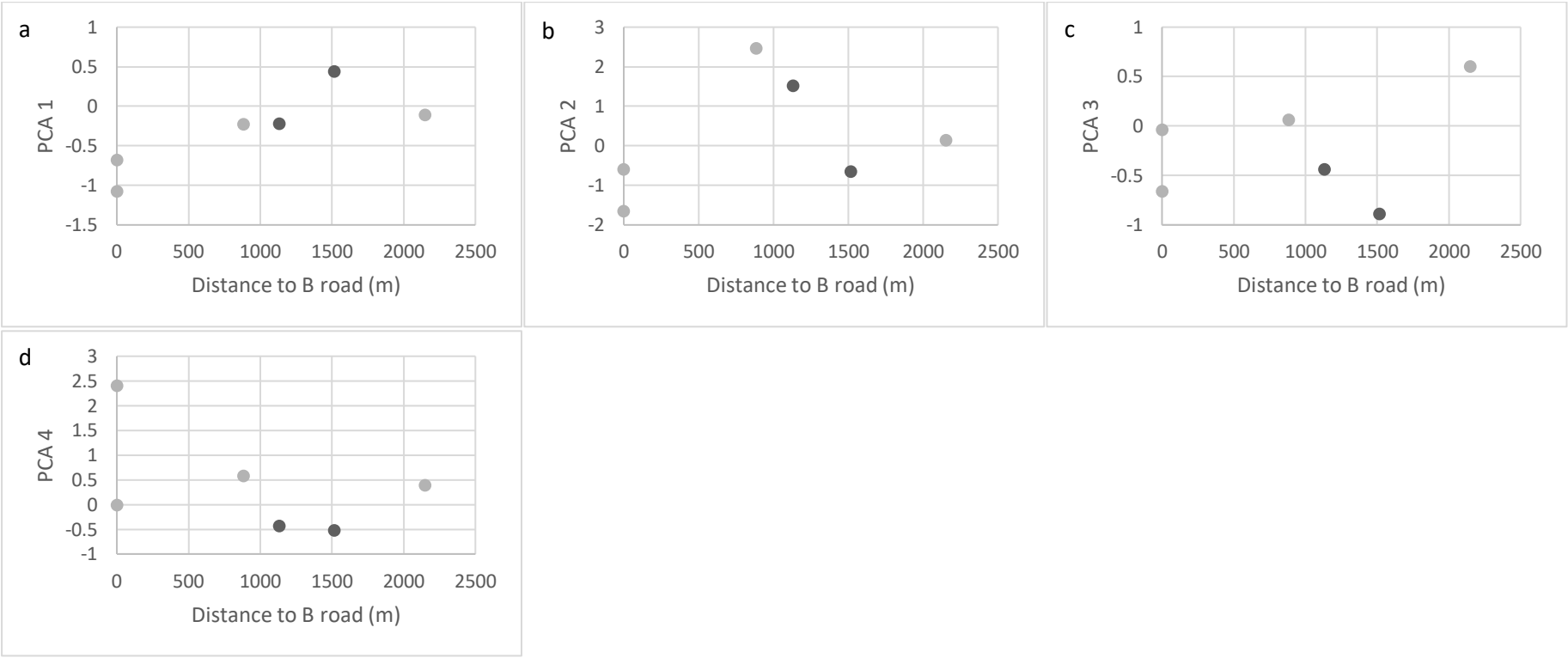
Appendix 4.23 Distance to nearest road of any type correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



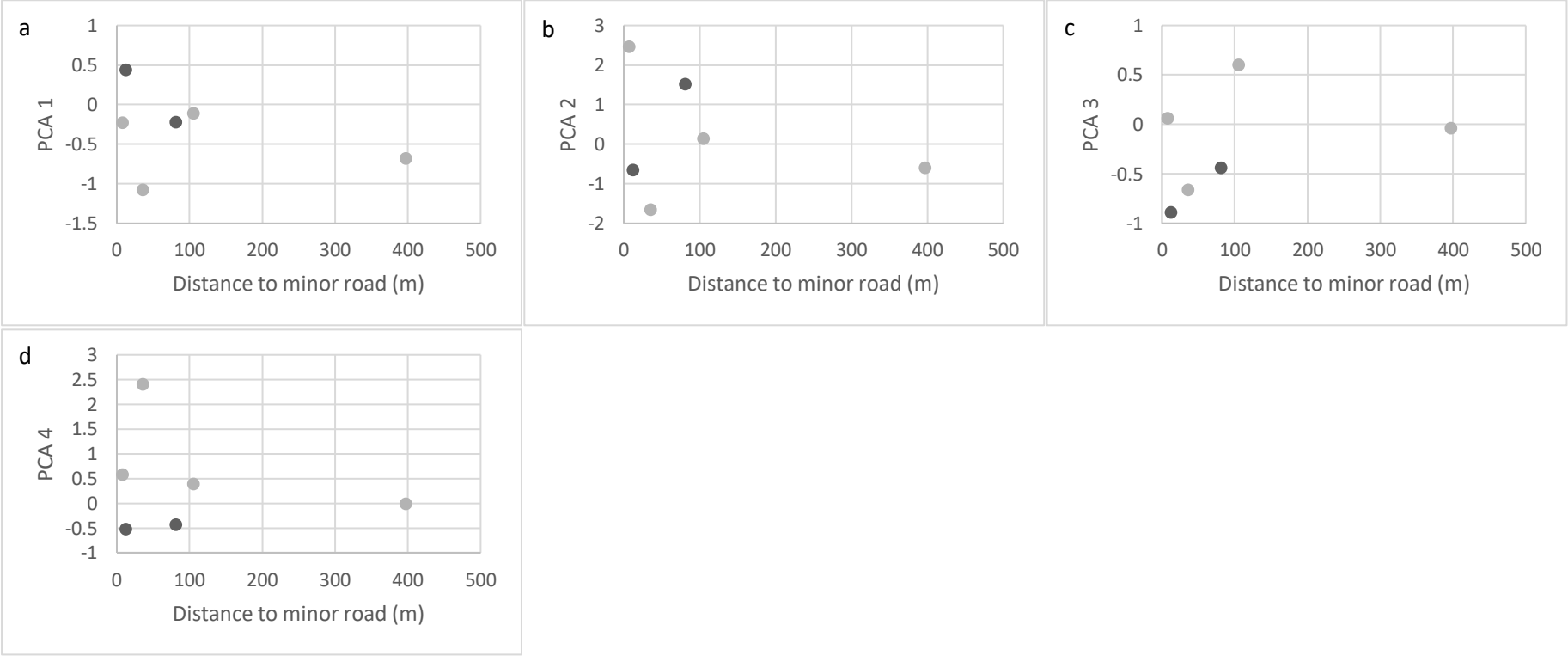
Appendix 4.24 Distance to nearest A road correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



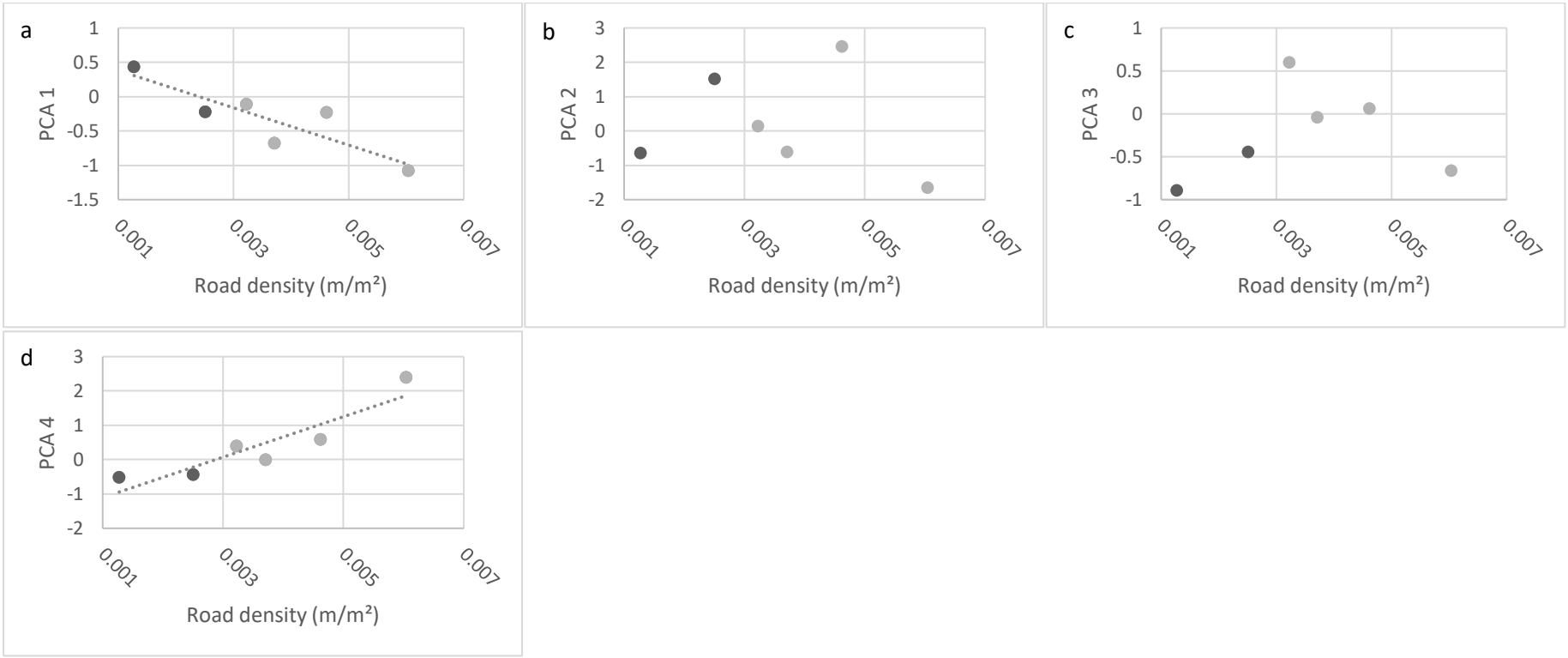
Appendix 4.25 Distance to nearest B road correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



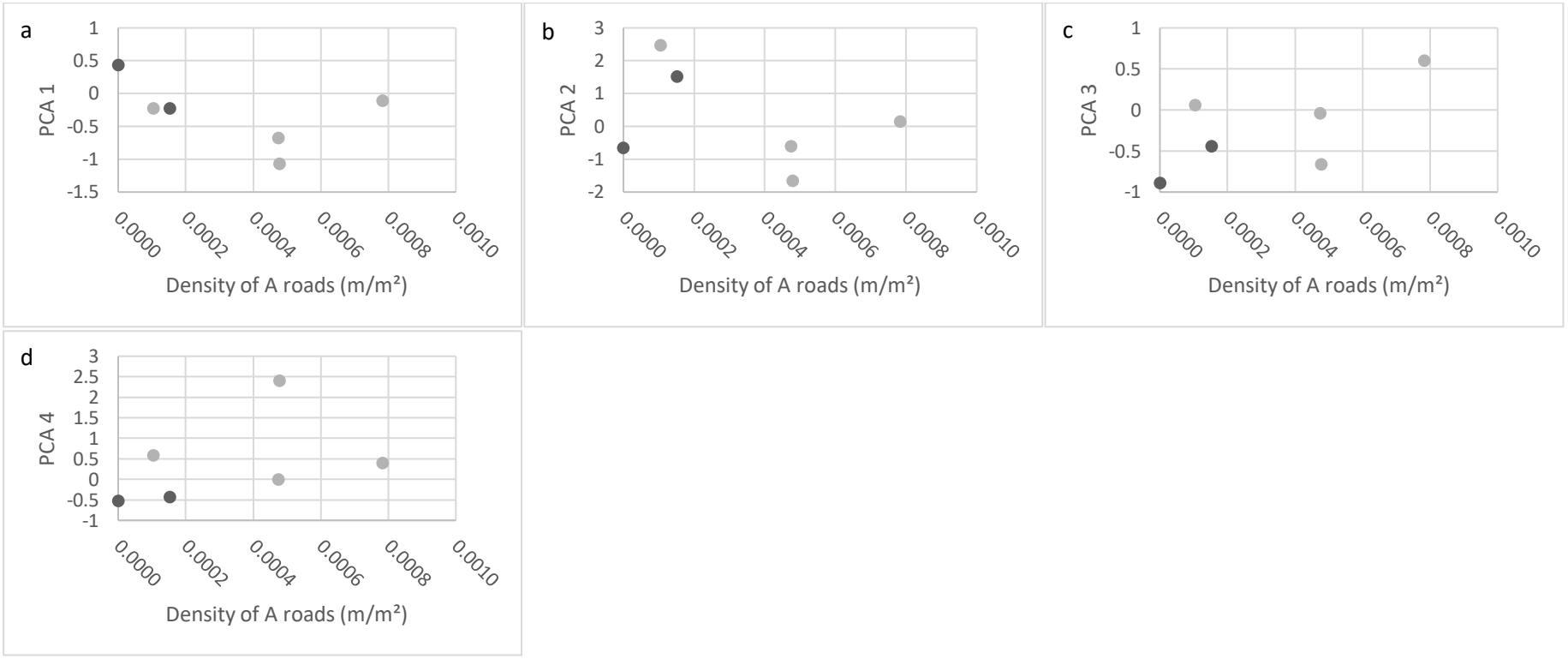
Appendix 4.26 Distance to nearest minor road correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



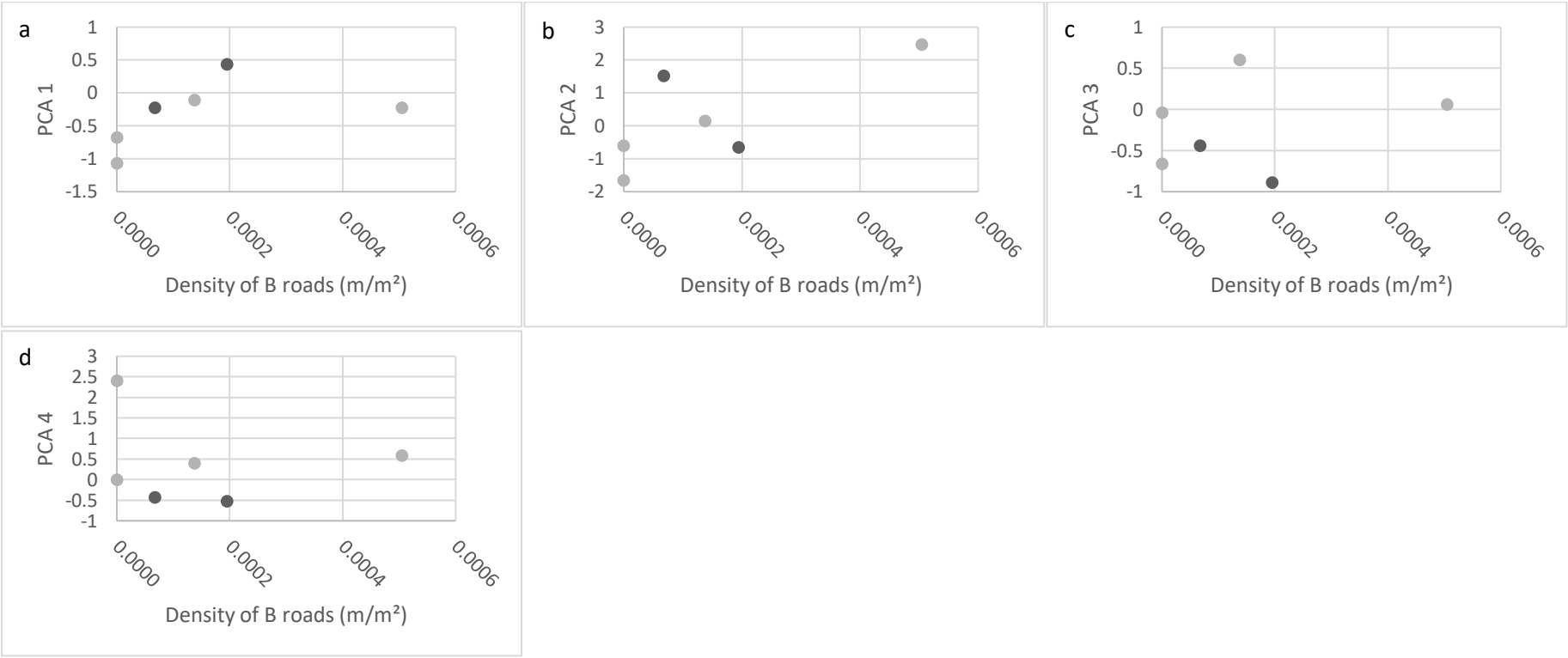
Appendix 4.27 Density of all types of road correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



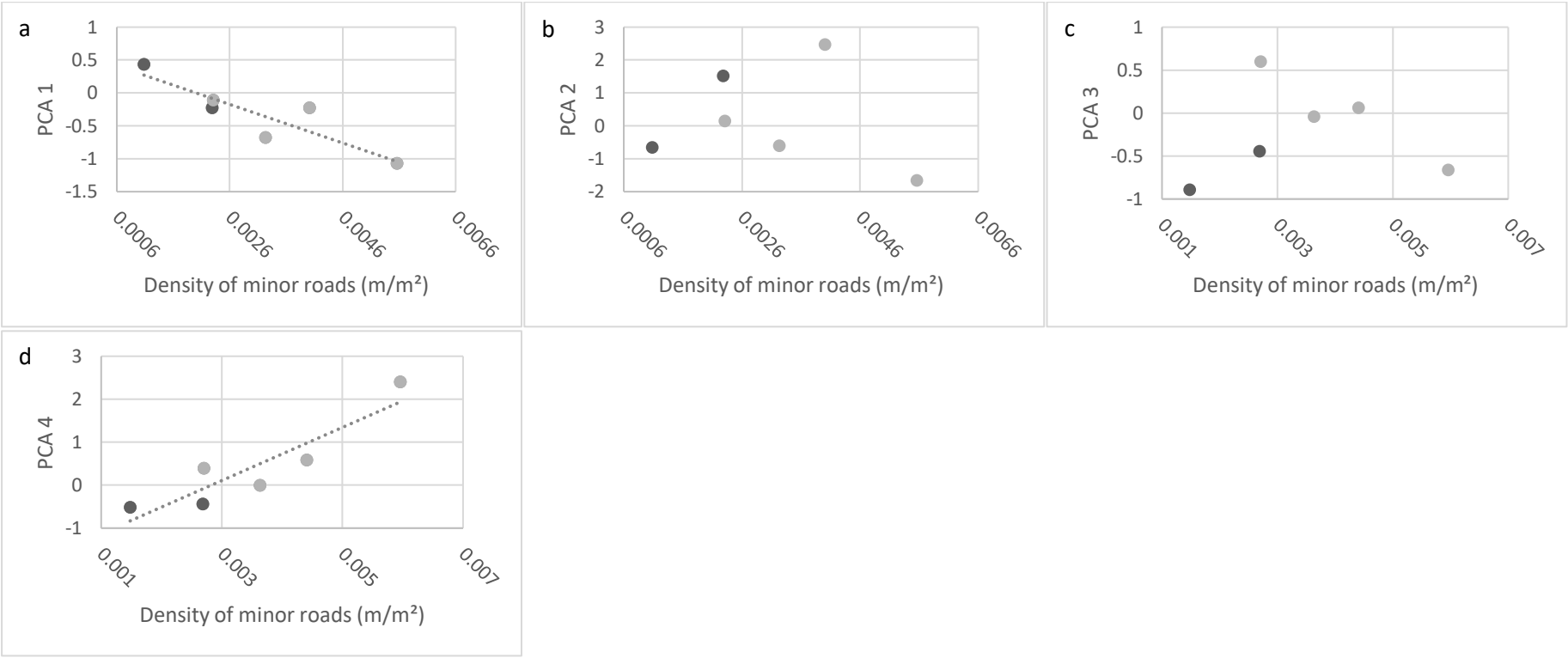
Appendix 4.28 Density of A roads correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



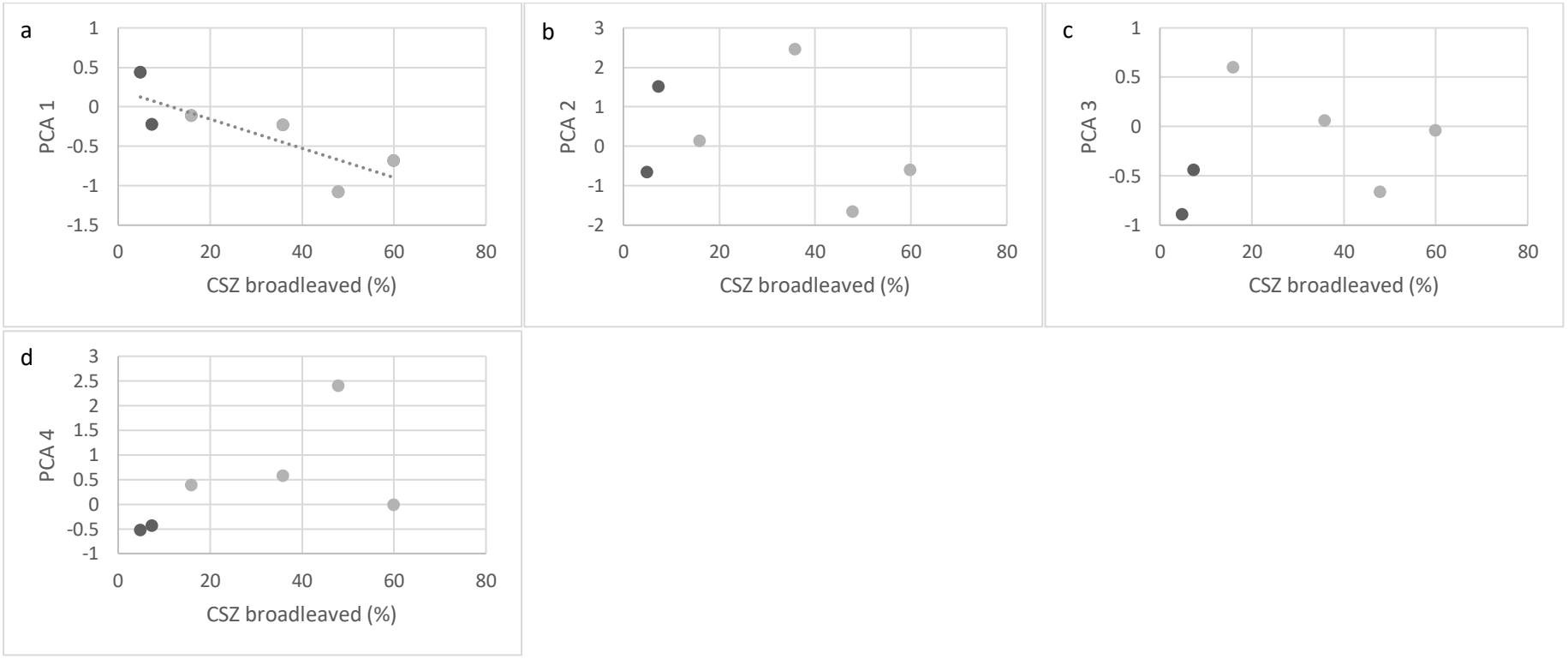
Appendix 4.29 Density of B roads correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



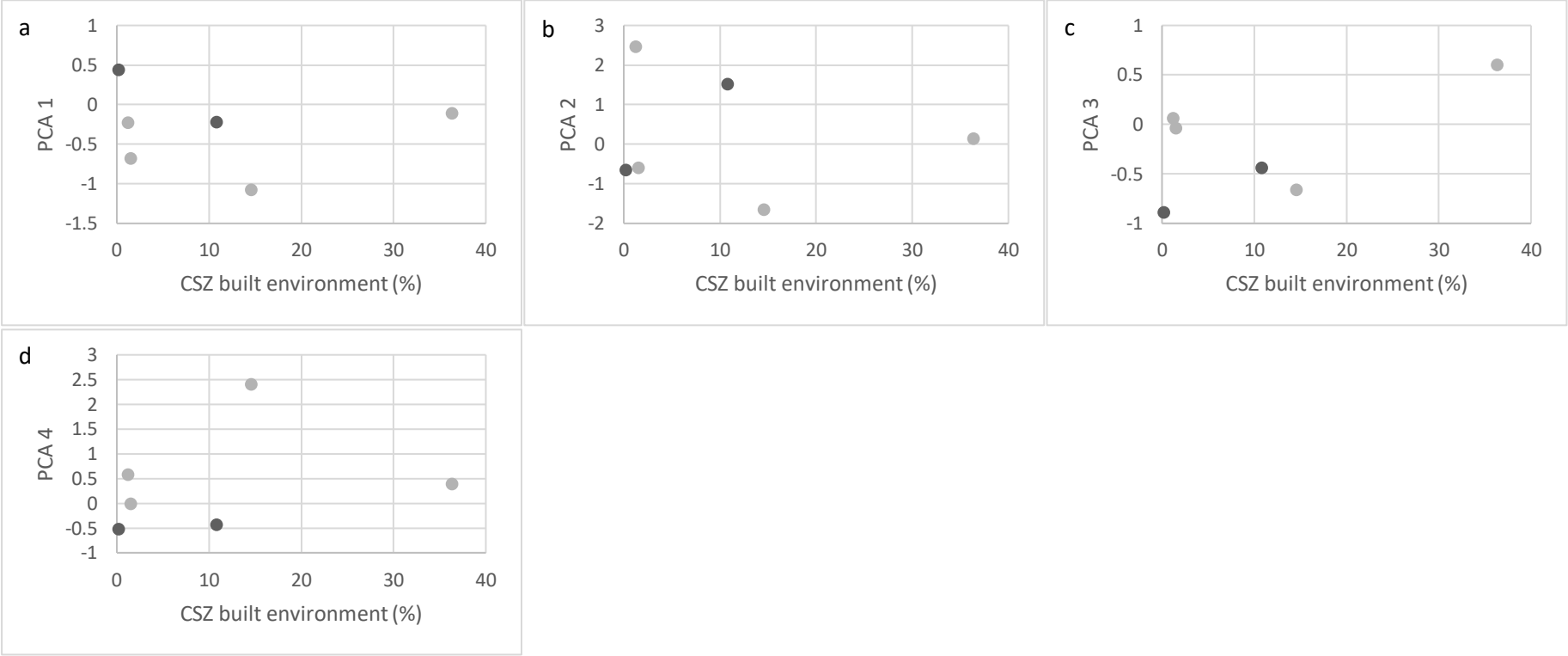
Appendix 4.30 Density of minor roads correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



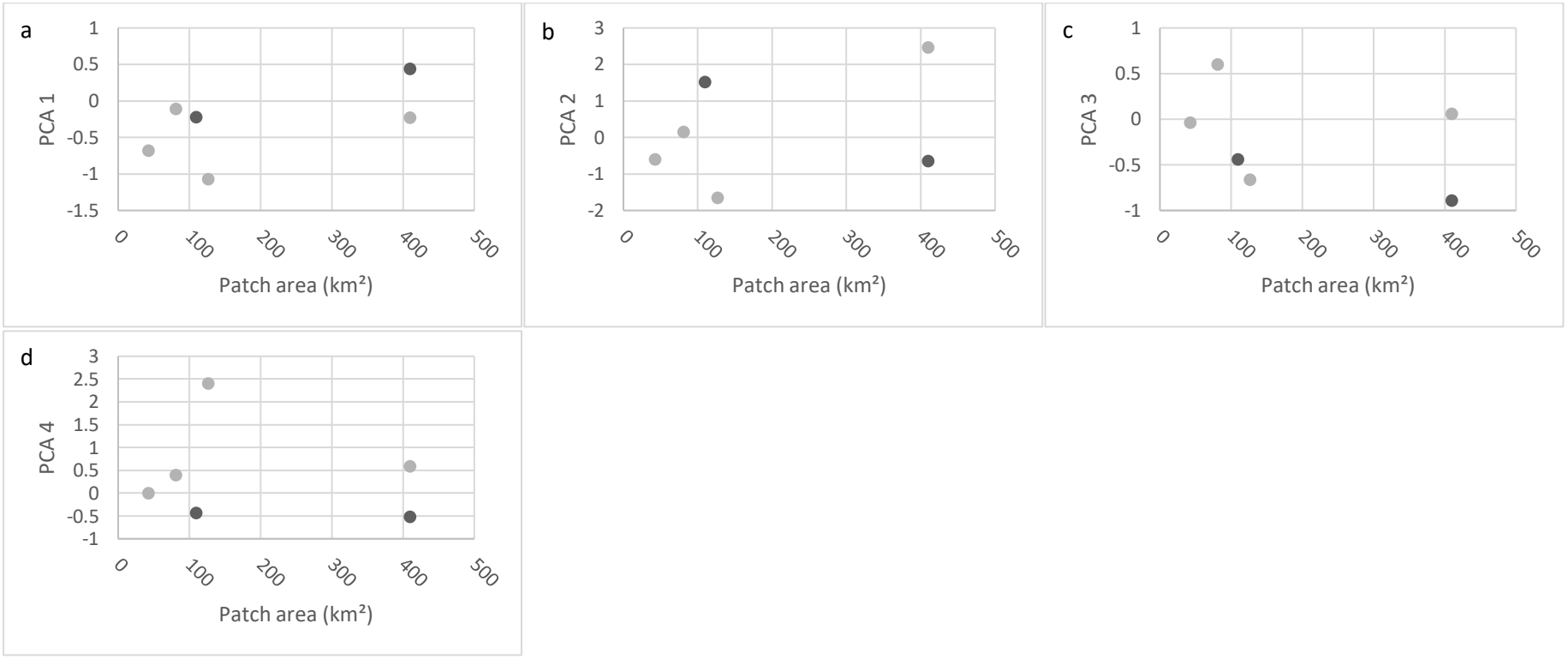
Appendix 4.31 CSZ broadleaved woodland (%) correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



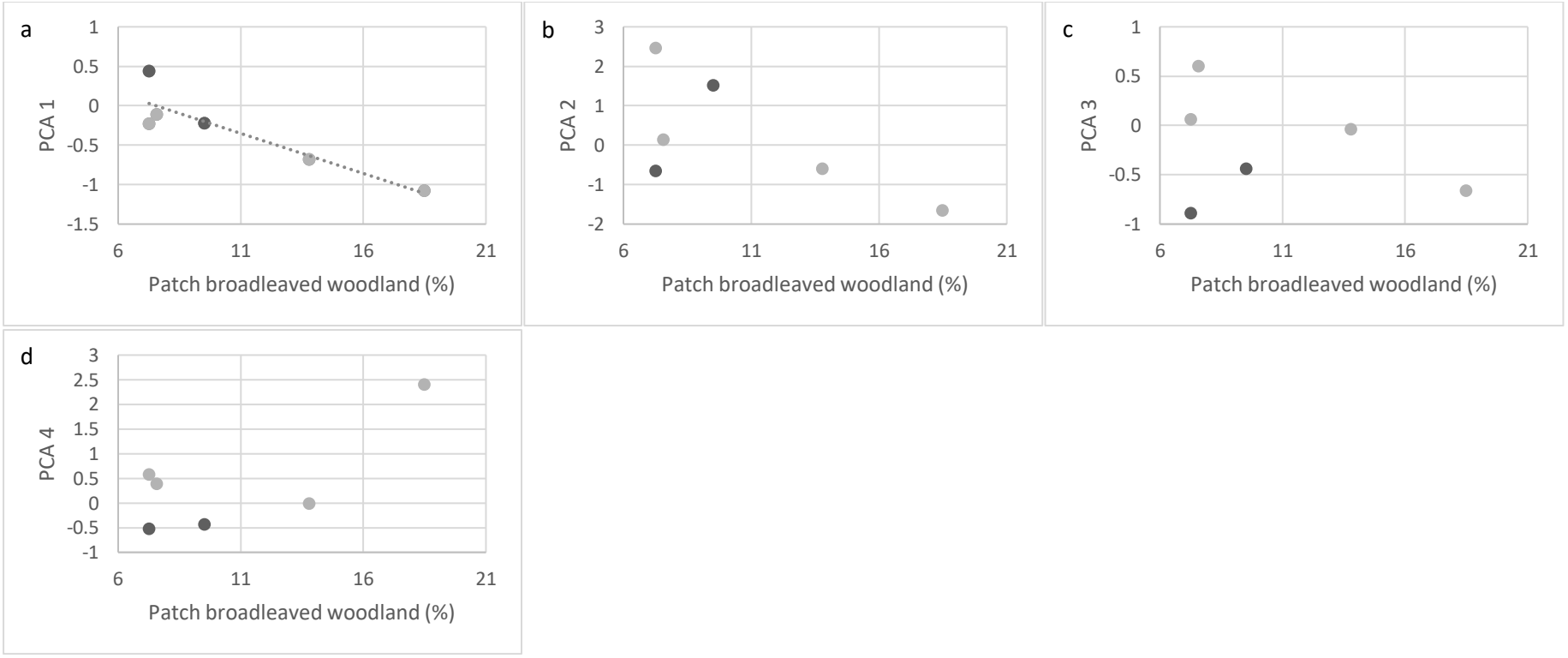
Appendix 4.32 CSZ built environment (%) correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



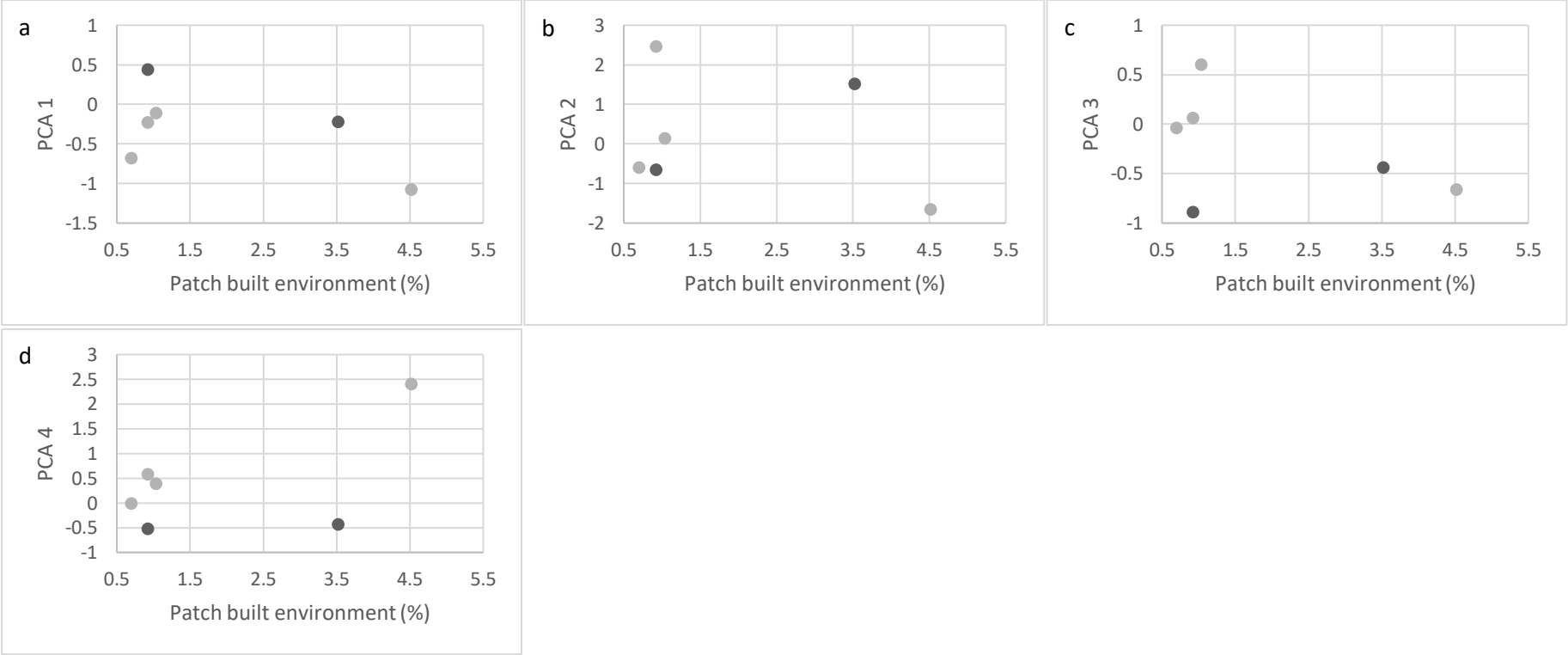
Appendix 4.33 Road defined patch area correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



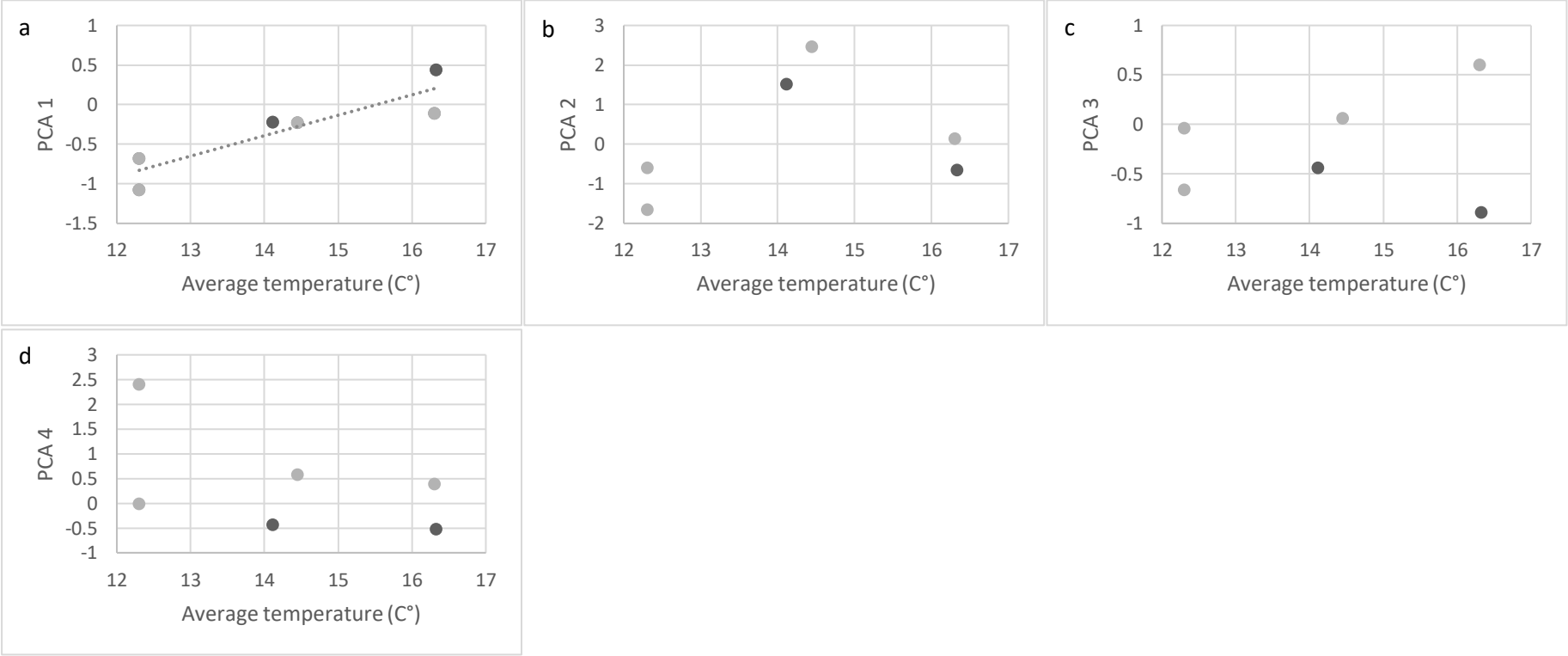
Appendix 4.34 The area of broadleaved woodland within road defined patches, as a percentage, correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



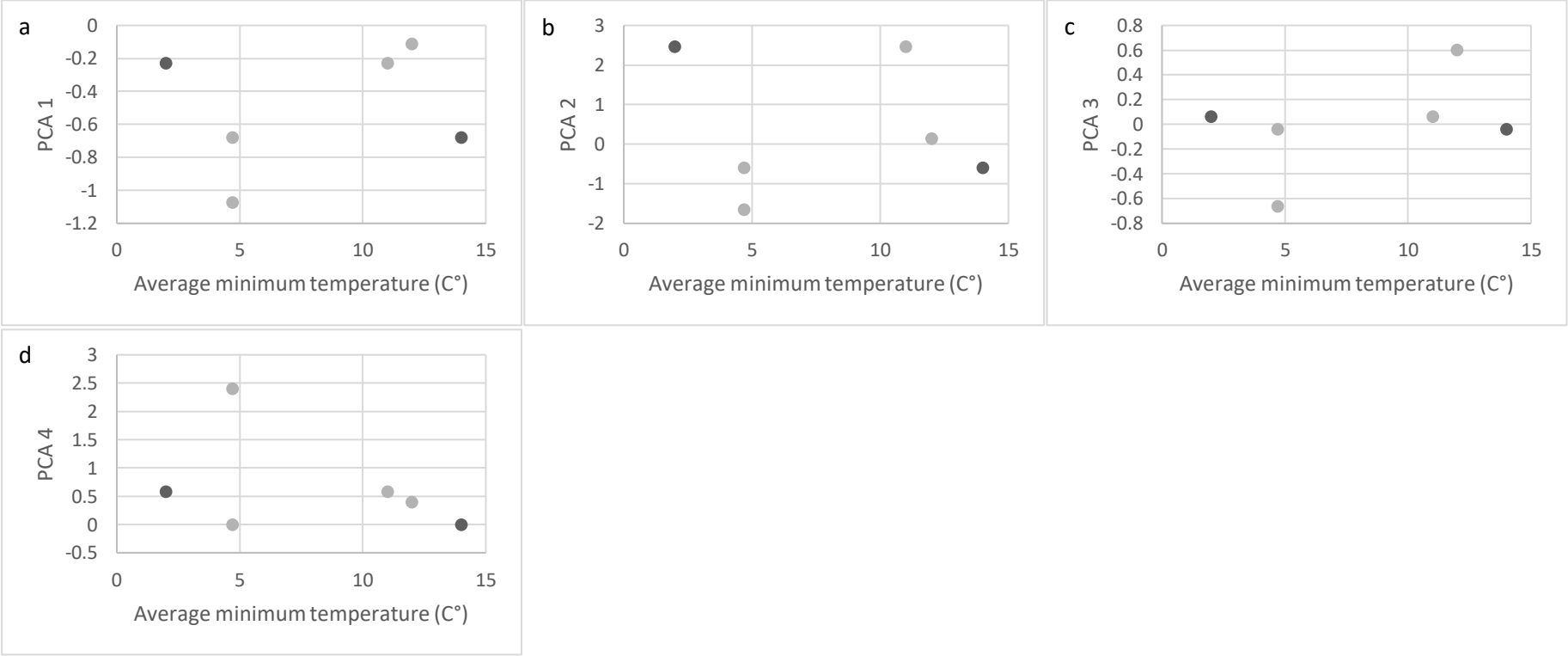
Appendix 4.35 The area of built environments within road defined patches as a percentage, correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



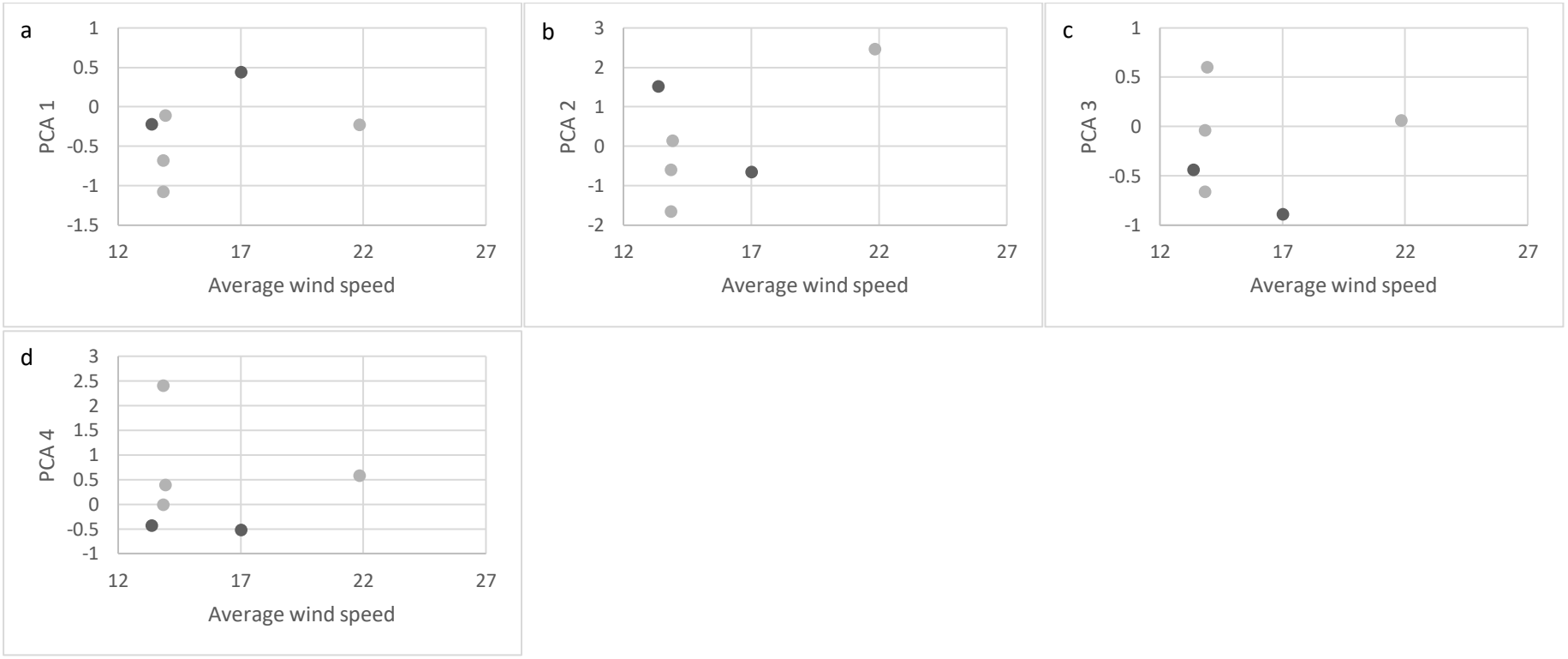
Appendix 4.36 Average temperature correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



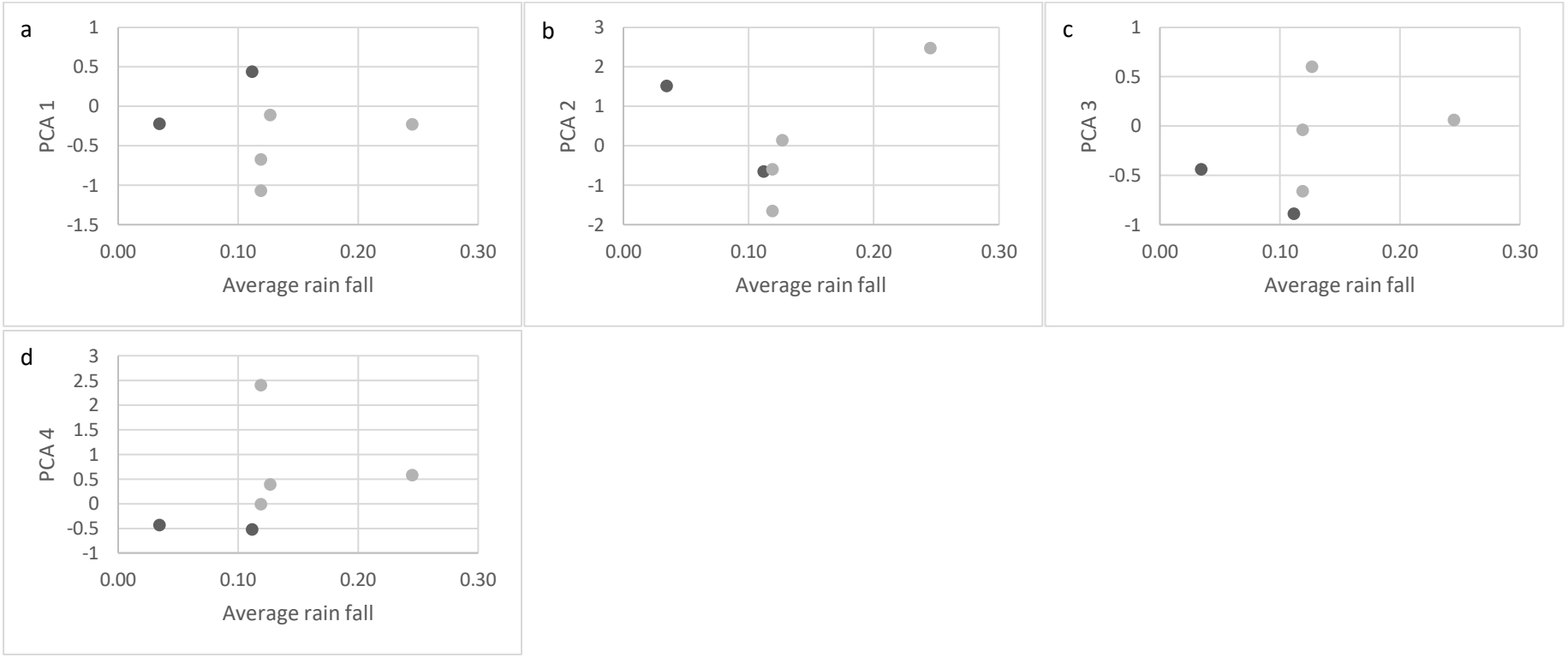
Appendix 4.37 Average minimum temperature correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



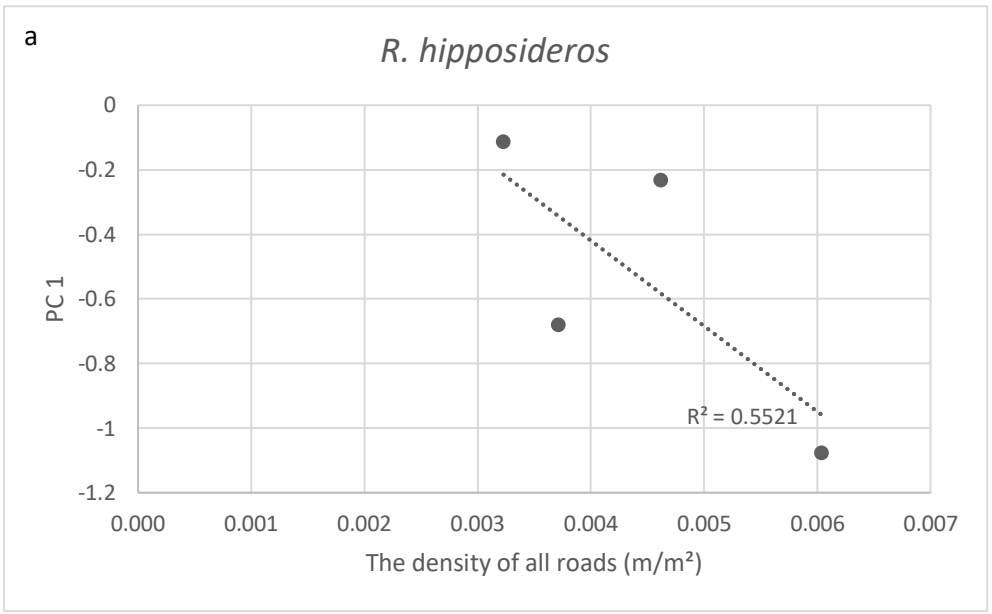
Appendix 4.38 Average wind speed correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).

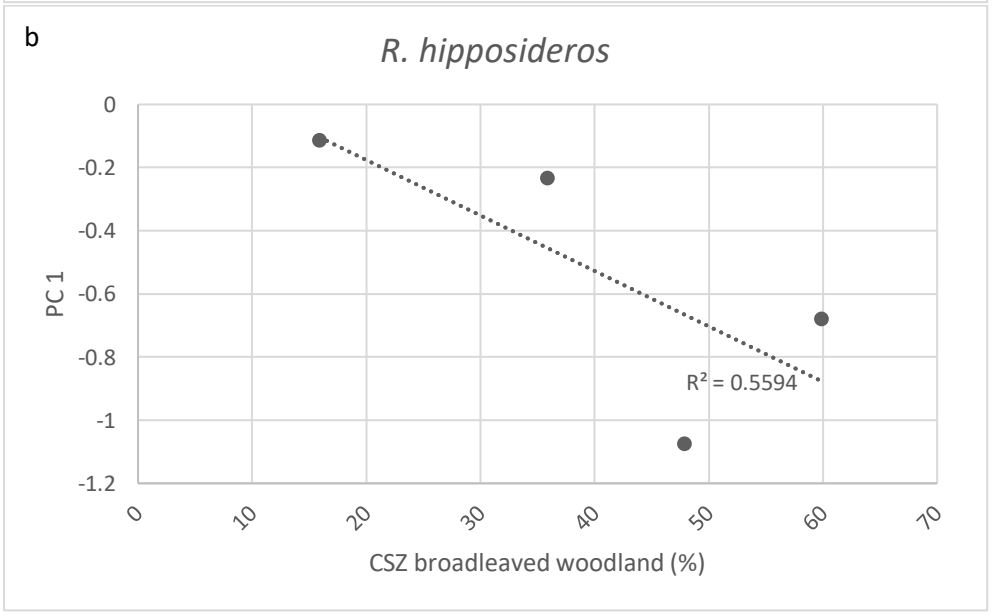


Appendix 4.39 Average rainfall events correlated against PC 1 (a), PC 2 (b), PC 3 (c) and PC 4 (d).



Appendix 4.40 Correlations between PC 1 and the density of all roads (a) and the percentage of broadleaved woodland within the CSZ (b) were moderately correlated but not significant (Pearson's correlation coefficient).



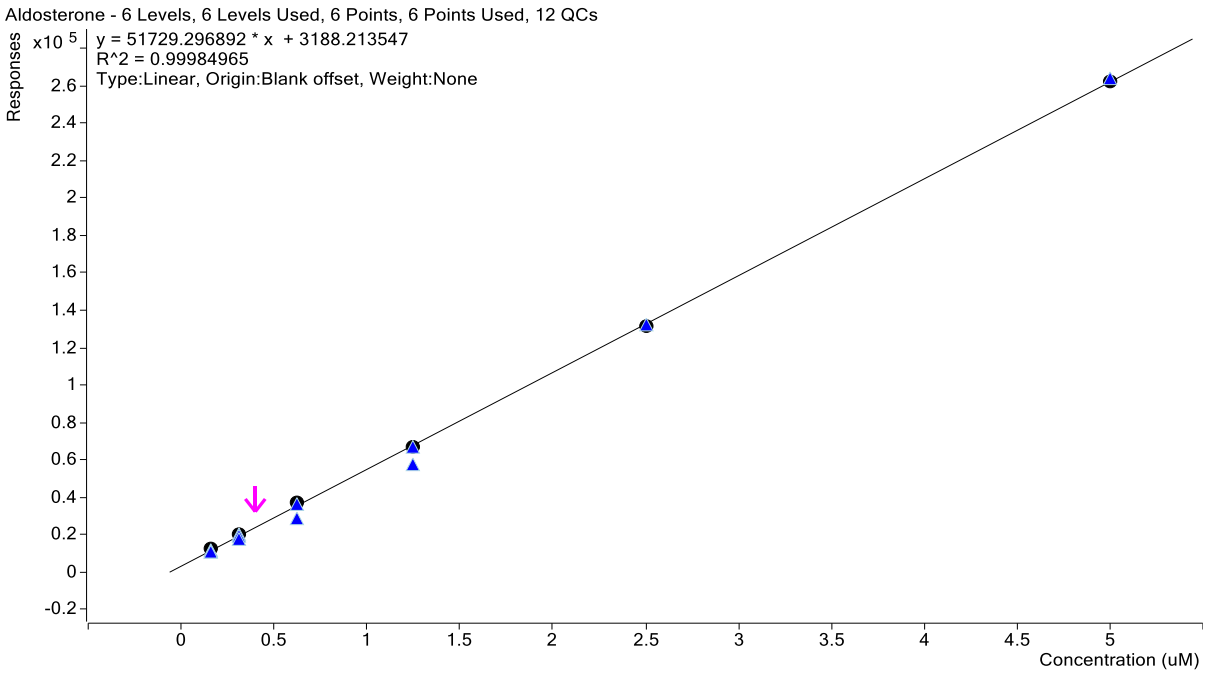


Appendix 4.41 The dates on which samples were collected. All samples were collected between June and September 2015 whilst bats roosted in maternity roosts.

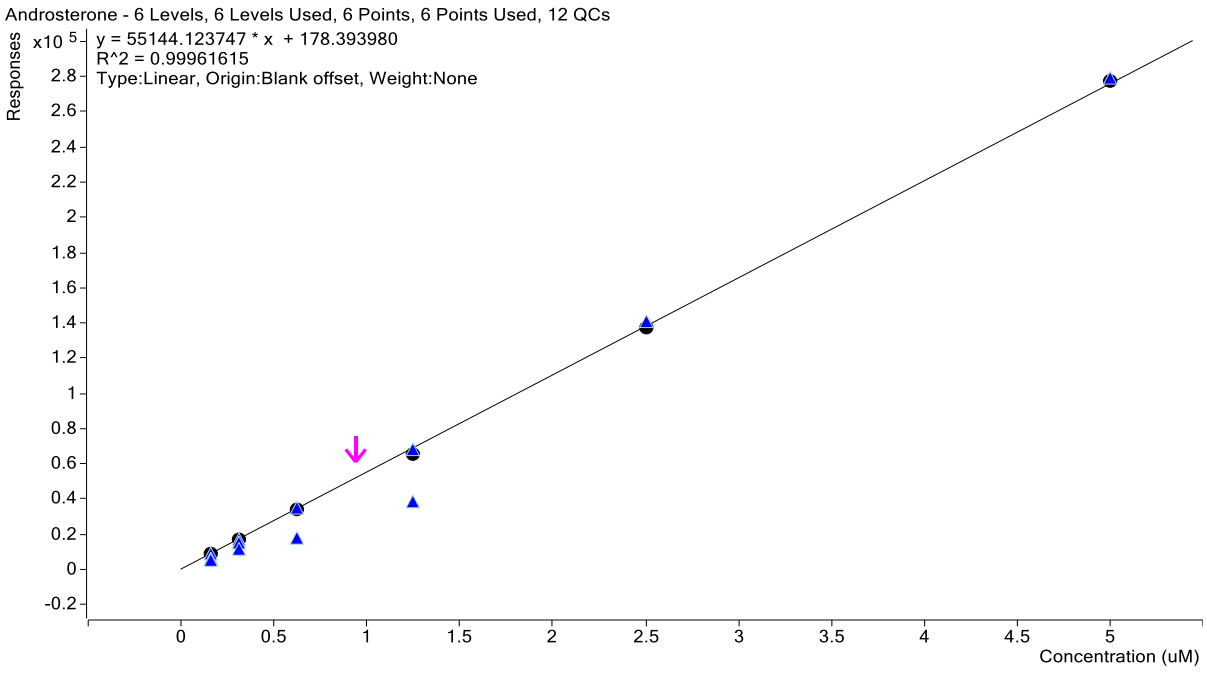
Site code	Species	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
1	*R. hipposideros*	28/07/2015	04/08/2015	11/08/2015	18/08/2015	26/08/2015
2	*R. hipposideros*	01/09/2015	21/09/2015	23/09/2015		
3	*R. hipposideros*	13/08/2015	20/08/2015	27/08/2015		
4	*P. auritus*	02/08/2015	08/08/2015	16/08/2015		
5	*P. auritus*	04/06/2015	28/07/2015	11/08/2015	18/08/2015	26/08/2015
6	*R. hipposideros*	21/08/2015	11/09/2015	23/09/2015		

Appendix 4.42 Calibration curves for aldosterone (a), androsterone (b), cortisone (c), corticosterone (d), dihydrotestosterone (e), hydrocortisone (f), pregnenalone (g), progesterone (h), testosterone (i), 1-dehydrotestosterone (j), 11-deoxycorticosterone (k), 17α-hydroxyprogesterone (l)

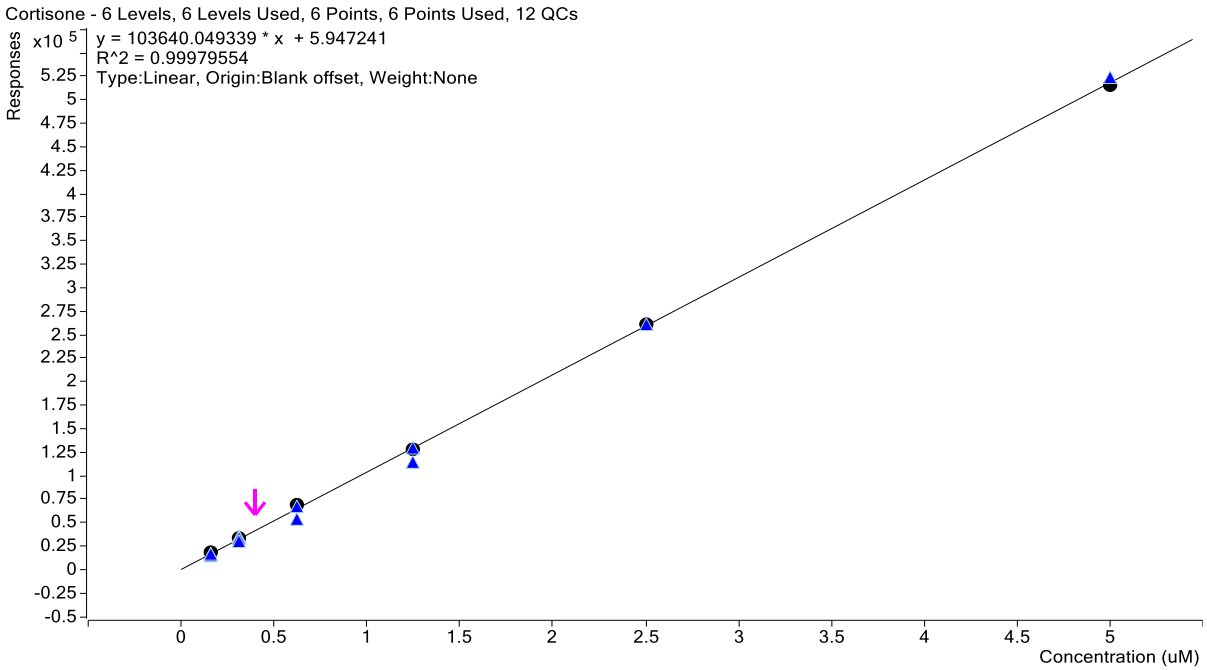
(a)



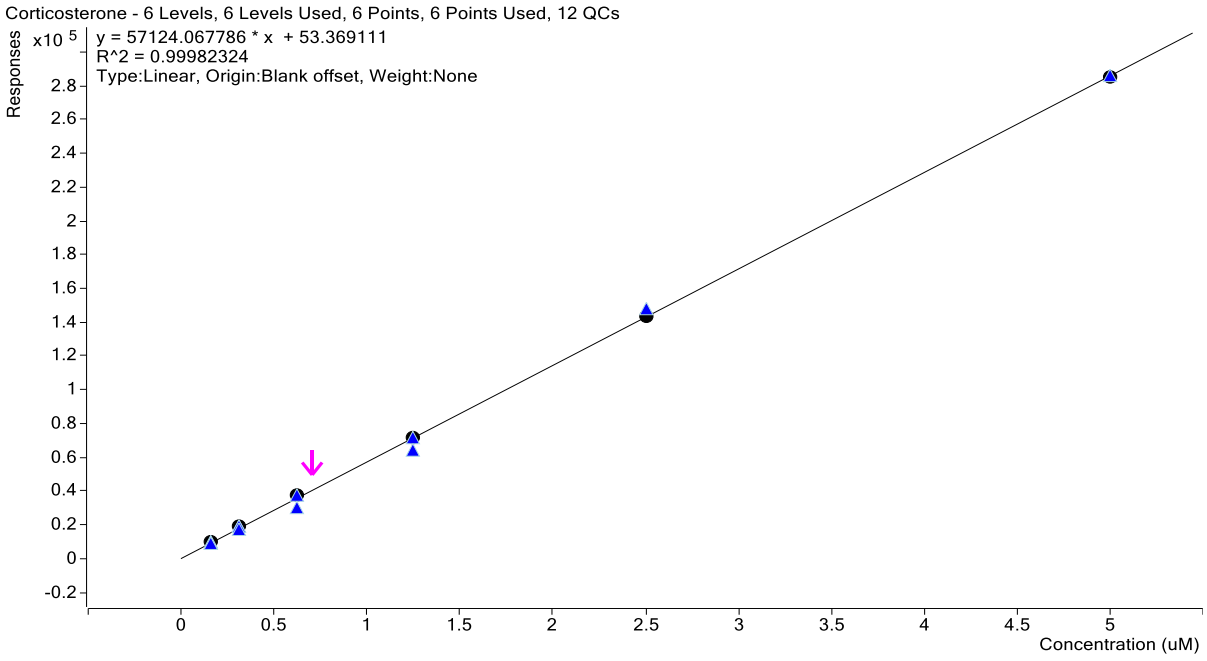
(b)



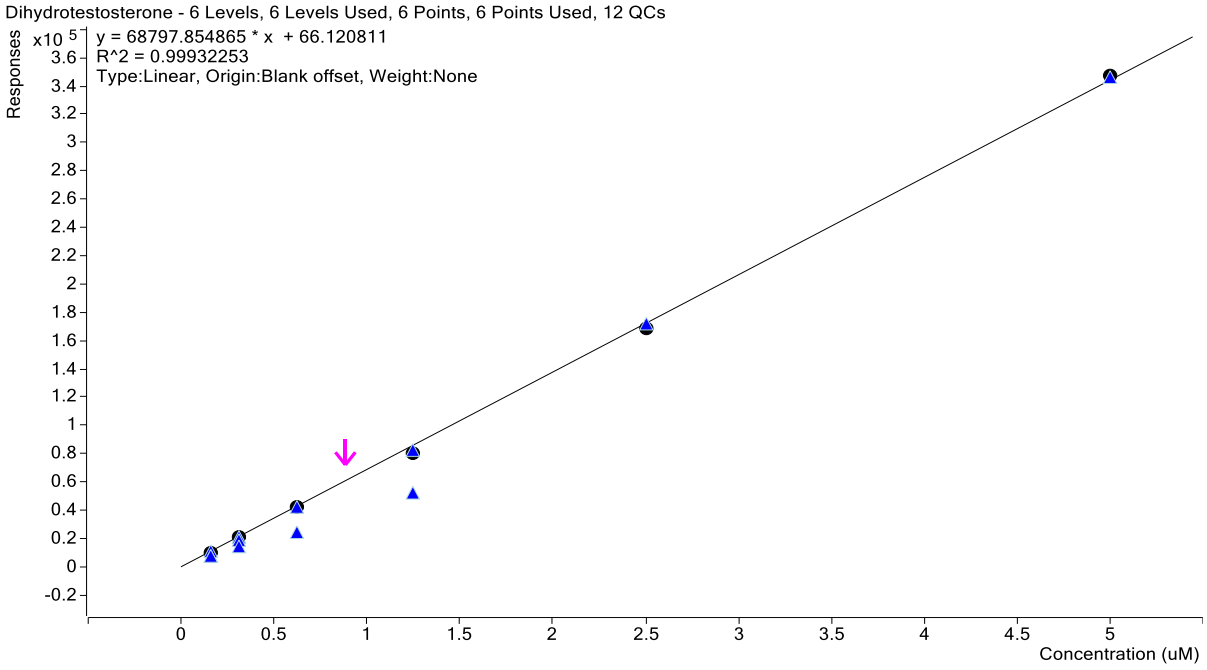
(c)


Cortisone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = 103640.049339 * x + 5.947241
R^2 = 0.99979554
Type:Linear, Origin:Blank offset, Weight:None
Responses
x10 5
Concentration (uM)

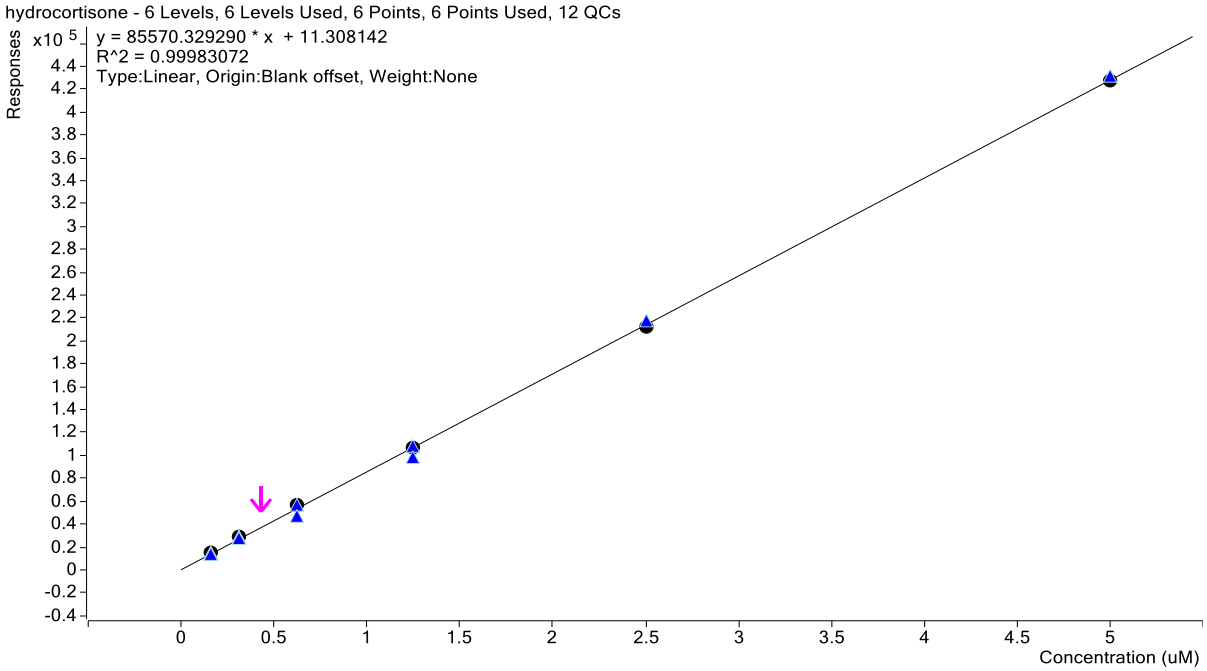
(d)


Corticosterone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = 57124.067786 * x + 53.369111
R^2 = 0.99982324
Type:Linear, Origin:Blank offset, Weight:None
Responses
x10 5
Concentration (uM)

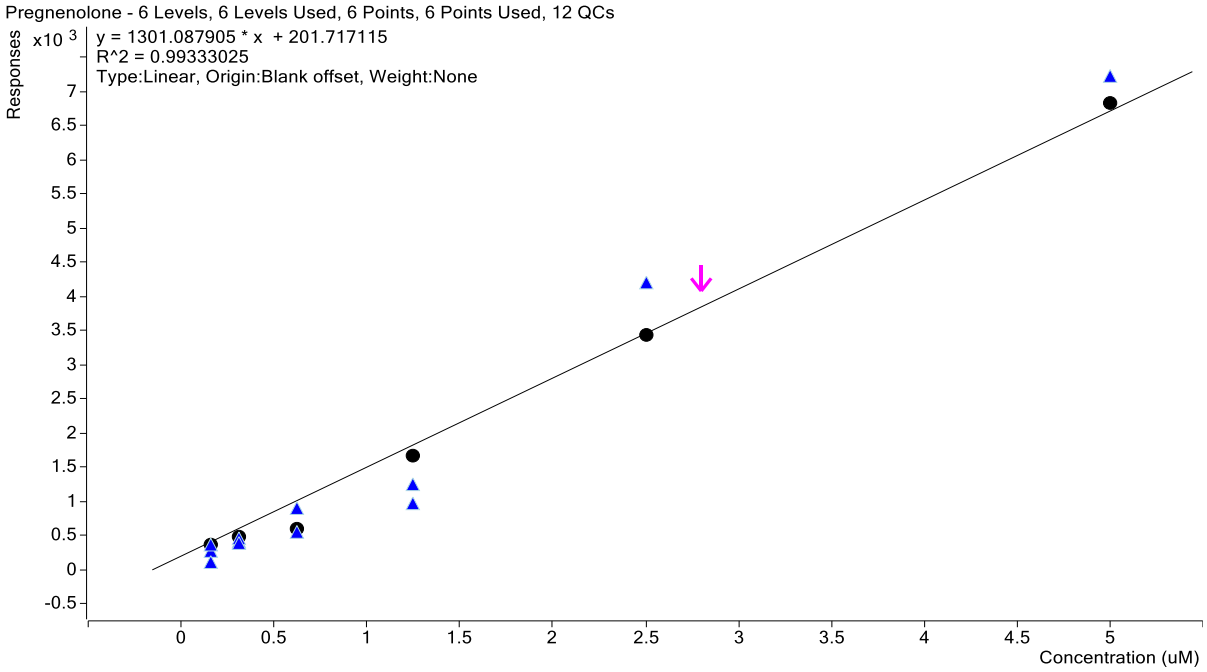
(e)


Dihydrotestosterone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = 68797.854865 * x + 66.120811
R^2 = 0.99932253
Type:Linear, Origin:Blank offset, Weight:None
Responses
x10 5
Concentration (uM)

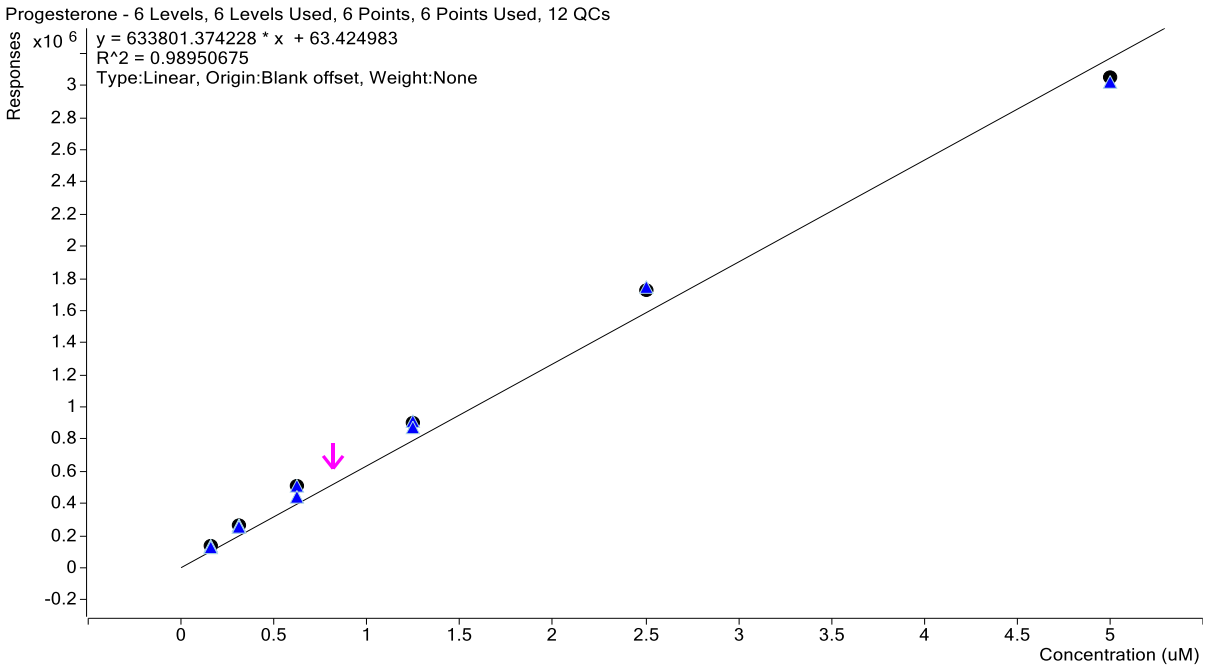
(f)


hydrocortisone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = 85570.329290 * x + 11.308142
R^2 = 0.99983072
Type:Linear, Origin:Blank offset, Weight:None
Responses
x10 5
Concentration (uM)

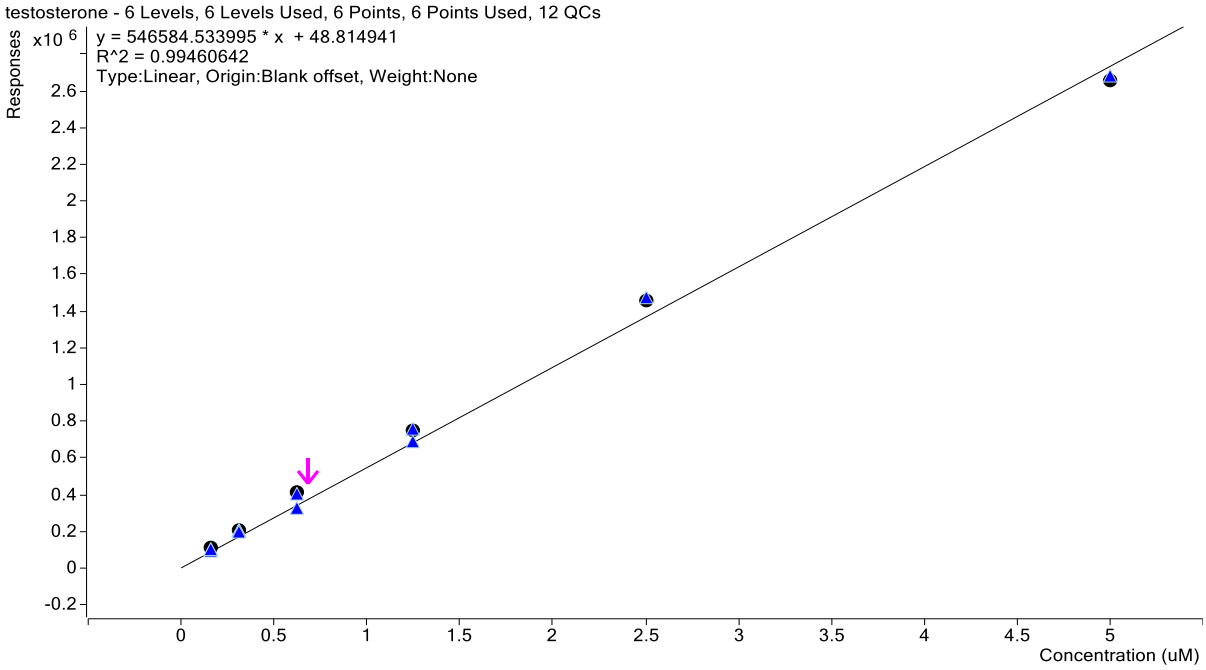
(g)


Pregnenolone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = 1301.087905 * x + 201.717115
R^2 = 0.99333025
Type:Linear, Origin:Blank offset, Weight:None
Responses
x10 3
Concentration (uM)

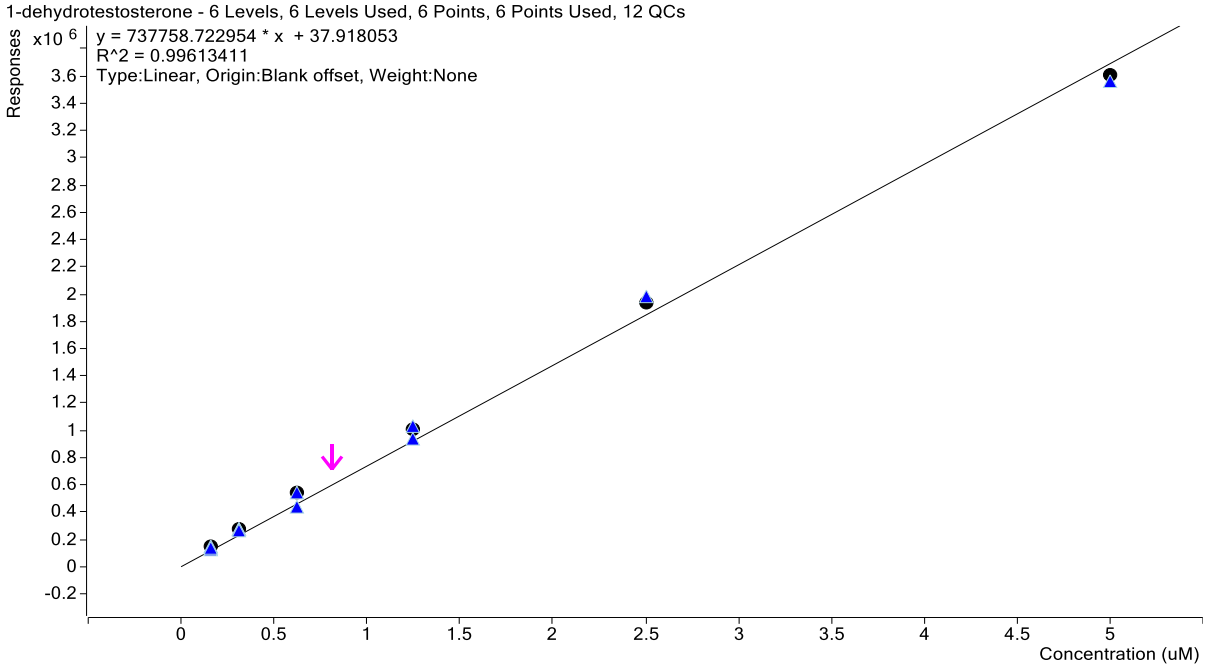
(h)


Progesterone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = 633801.374228 * x + 63.424983
R^2 = 0.98950675
Type:Linear, Origin:Blank offset, Weight:None
Responses
x10 6
Concentration (uM)

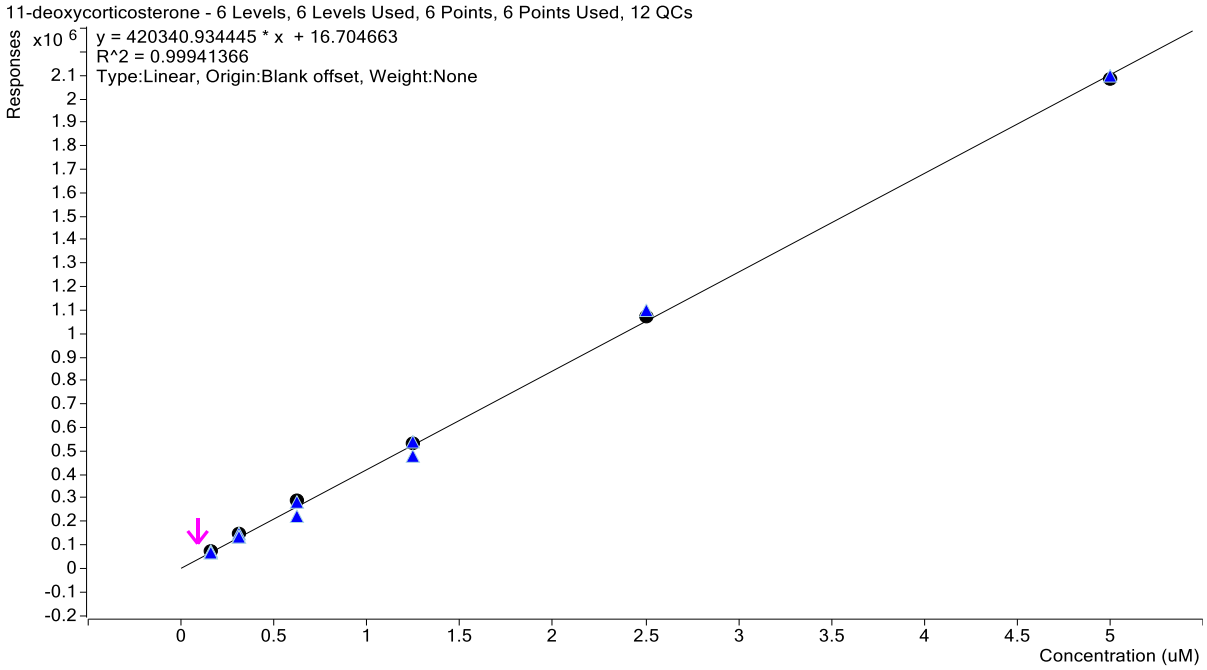
(i)


testosterone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = 546584.533995 * x + 48.814941
R^2 = 0.99460642
Type:Linear, Origin:Blank offset, Weight:None
Responses
x10 6
Concentration (uM)

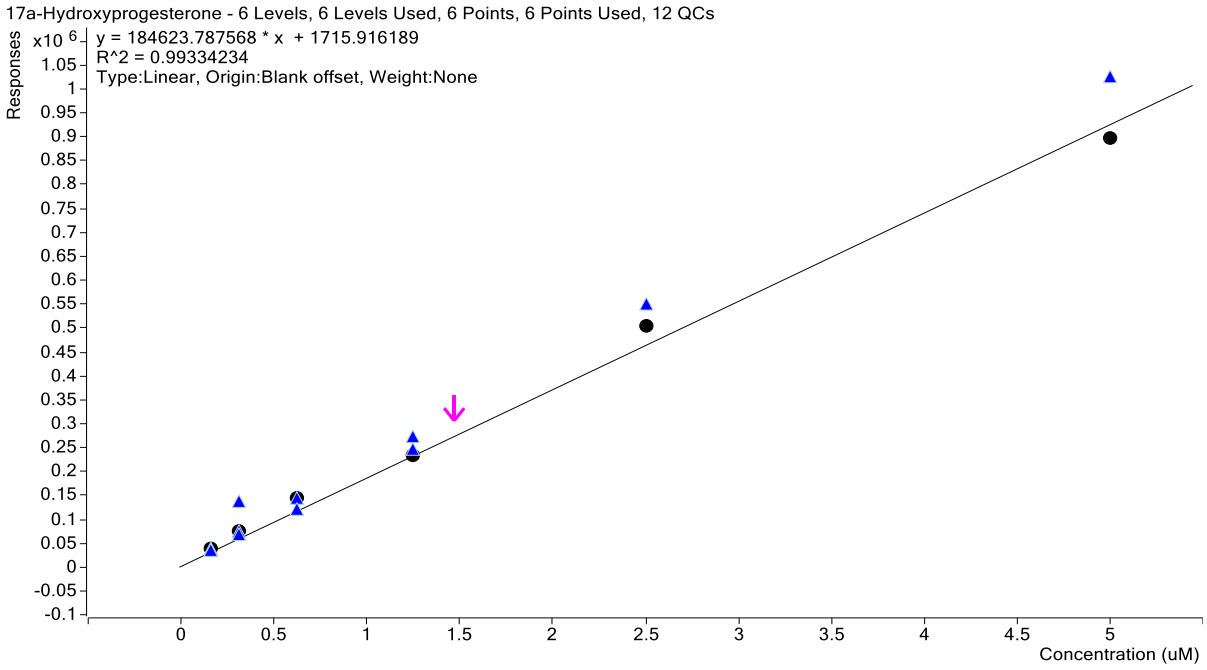
(j)


1-dehydrotestosterone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = 737758.722954 * x + 37.918053
R^2 = 0.99613411
Type:Linear, Origin:Blank offset, Weight:None
Responses
x10 6
Concentration (uM)

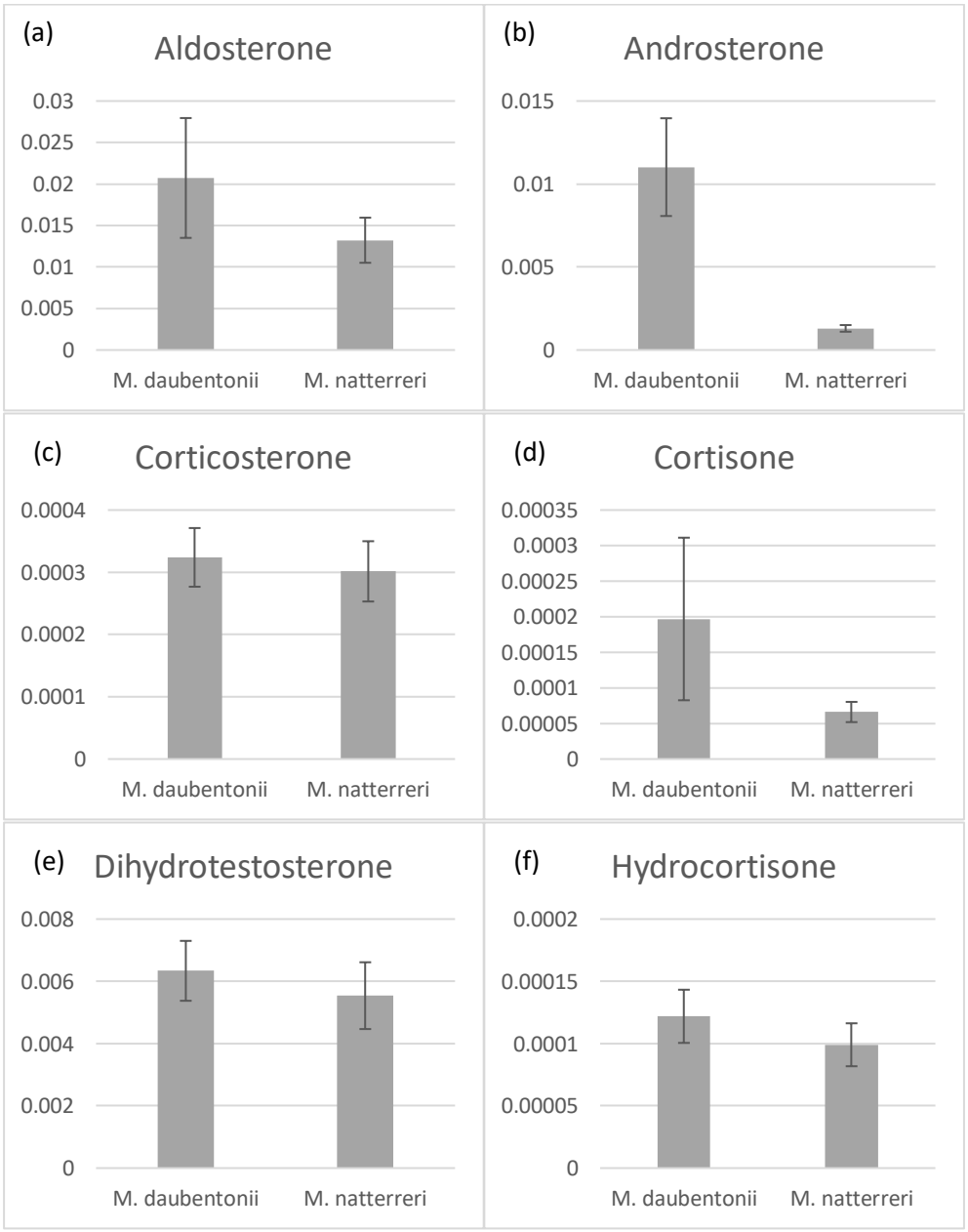
(k)

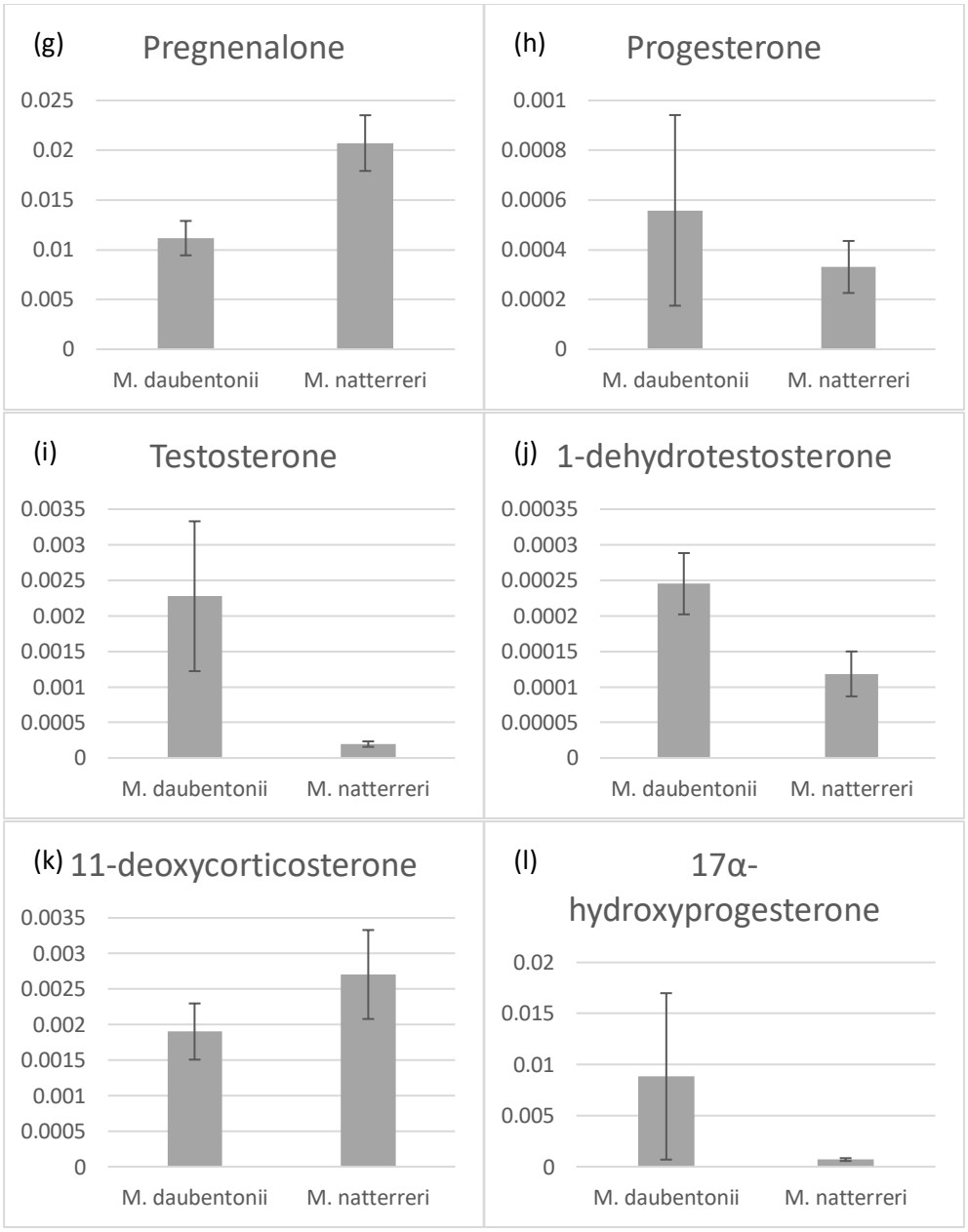

11-deoxycorticosterone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = 420340.934445 * x + 16.704663
R^2 = 0.99941366
Type:Linear, Origin:Blank offset, Weight:None
Responses
x10 6
Concentration (uM)

(l)

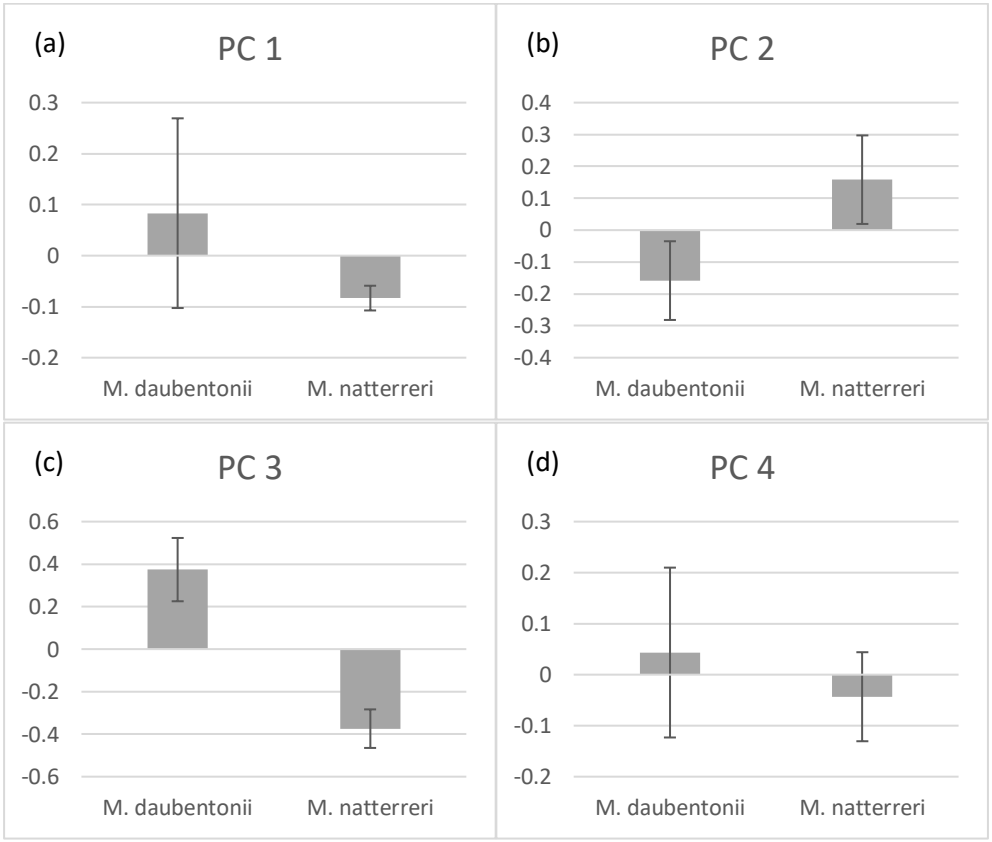

17a-Hydroxyprogesterone - 6 Levels, 6 Levels Used, 6 Points, 6 Points Used, 12 QCs
y = 184623.787568 * x + 1715.916189
R^2 = 0.99334234
Type:Linear, Origin:Blank offset, Weight:None
Responses
x10 6
Concentration (uM)

Appendix 4.43 Comparisons between *M. daubentonii* and *M. nattereri* average concentrations (µM/mg) of target compounds; aldosterone (a), androsterone (b), corticosterone (c), cortisone (d), dihydrosterone (e), hydrocortisone (f), pregnenolone (g), progesterone (h), testosterone (i), 1-dehydrotestosterone (j), 11-deoxycorticosterone (k), 17α-hydroxyprogesterone (l).

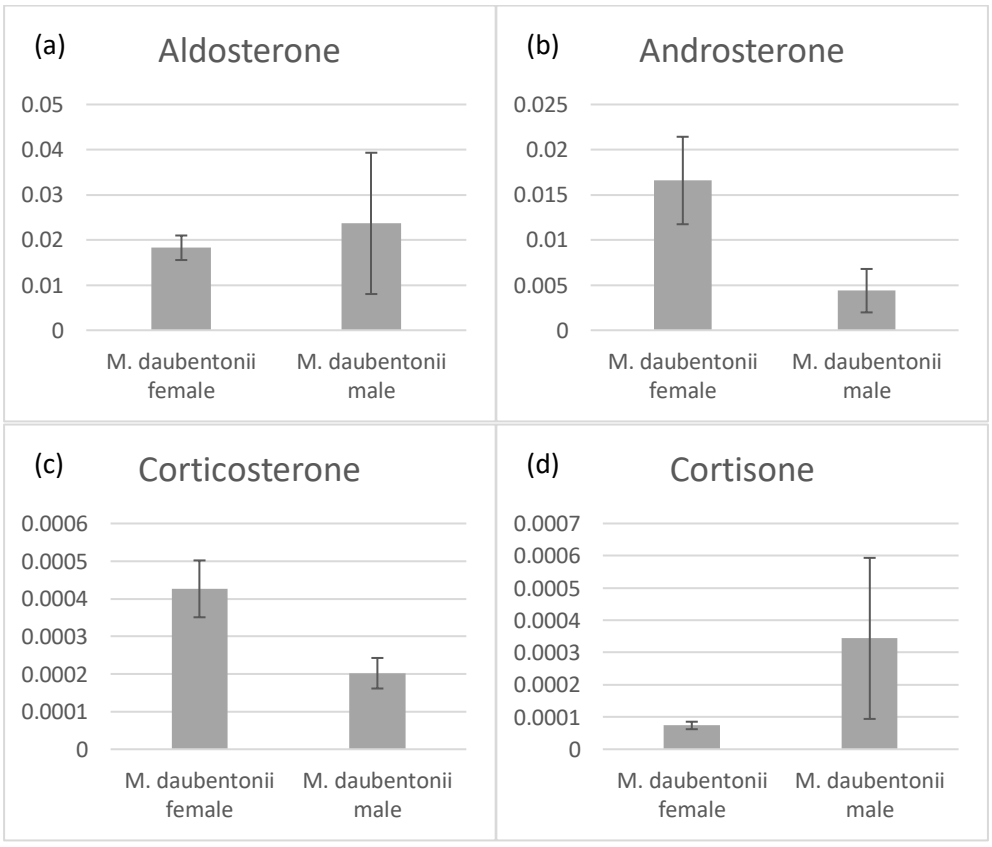


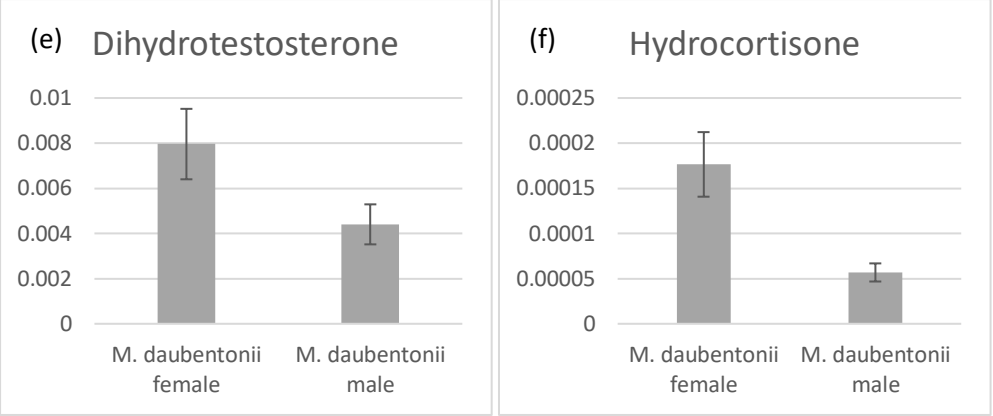

(g)
Pregnenalone
0.025
0.02
0.015
0.01
0.005
0
M. daubentonii
M. natterreri
(h)
Progesterone
0.001
0.0008
0.0006
0.0004
0.0002
0
M. daubentonii
M. natterreri
(i)
Testosterone
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
M. daubentonii
M. natterreri
(j)
1-dehydrotestosterone
0.00035
0.0003
0.00025
0.0002
0.00015
0.0001
0.00005
0
M. daubentonii
M. natterreri
(k)
11-deoxycorticosterone
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
M. daubentonii
M. natterreri
(l)
17α-hydroxyprogesterone
0.02
0.015
0.01
0.005
0
M. daubentonii
M. natterreri

Appendix 4.44 Comparisons between *M. daubentonii* and *M. nattereri;* PC 1 (a), PC 2 (b), PC 3 (c), PC 4 (d).

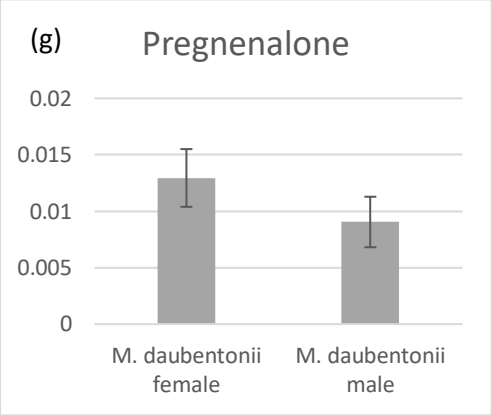


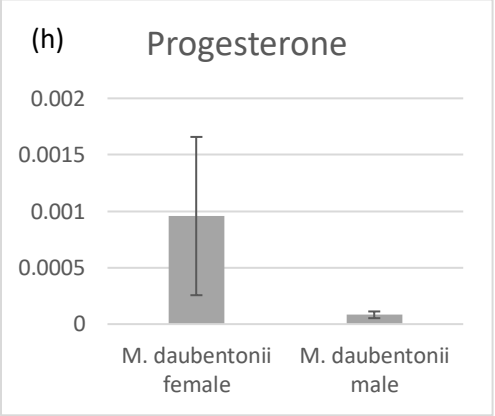
Appendix 4.45 Comparisons between. *M. daubentonii* and *M. nattereri* males and females for each target compound (µM/mg); *M. daubentonii* aldosterone (a), androsterone (b), corticosterone (c), cortisone (d), dihydrotestosterone (e), hydrocortisone (f), Pregnenolone (g), progesterone (h), testosterone (i), 1-dehydrotestosterone (j), 11-deoxycorticosterone (k), 17α-hydroxyprogesterone (l); *M. nattereri*, aldosterone (m), androsterone (n), corticosterone (o), cortisone (p), dihydrotestosterone (q), hydrocortisone (r), Pregnenolone (s), progesterone (t), testosterone (u), 1-dehydrotestosterone (v), 11-deoxycorticosterone (w), 17α-hydroxyprogesterone (x).

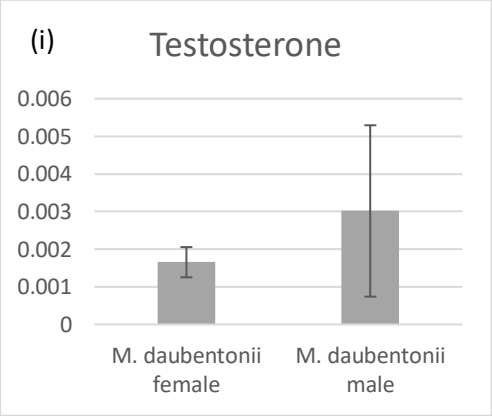


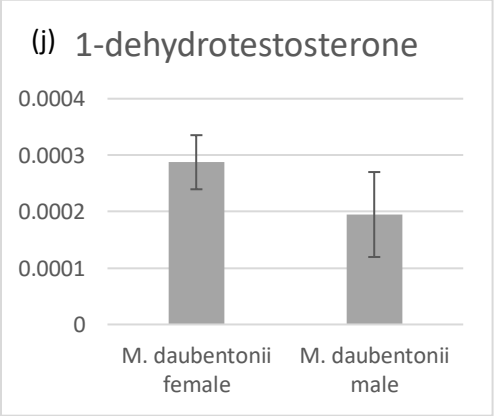

(e) Dihydrotestosterone
0.01
0.008
0.006
0.004
0.002
0
M. daubentonii female
M. daubentonii male

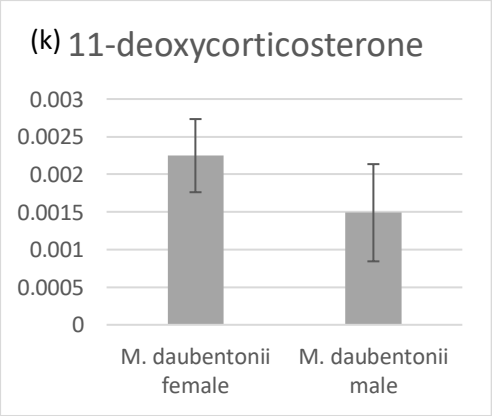
(f) Hydrocortisone
0.00025
0.0002
0.00015
0.0001
0.00005
0
M. daubentonii female
M. daubentonii male

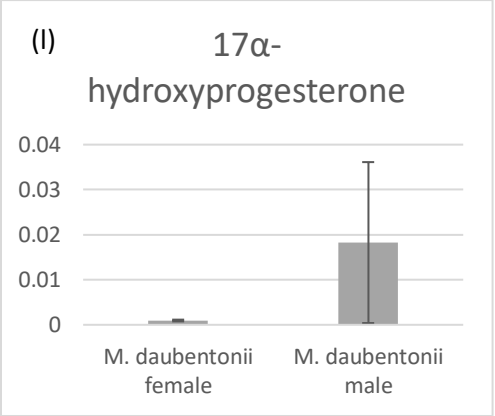

(g) Pregnenalone
0.02
0.015
0.01
0.005
0
M. daubentonii female
M. daubentonii male

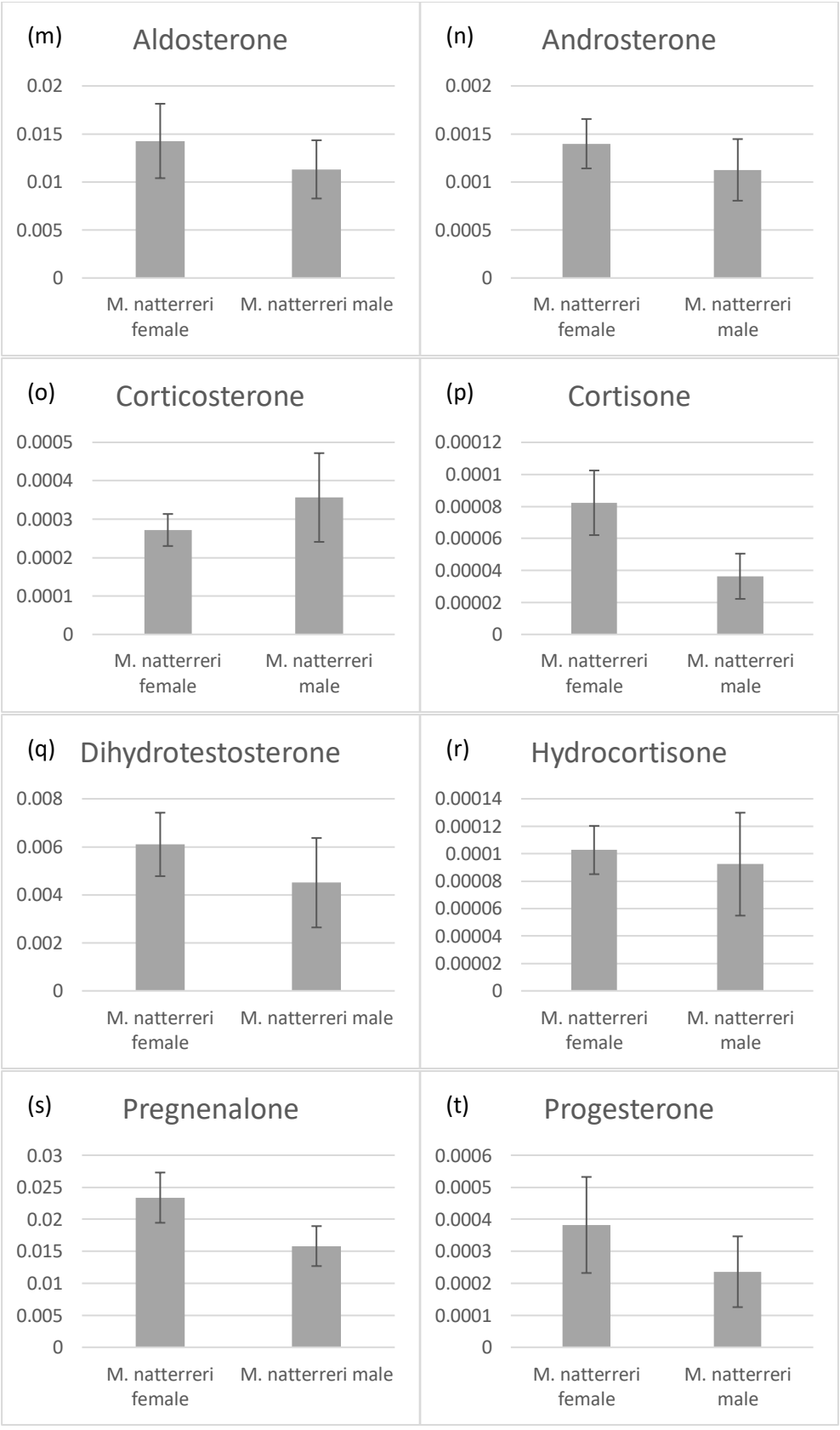

(h) Progesterone
0.002
0.0015
0.001
0.0005
0
M. daubentonii female
M. daubentonii male

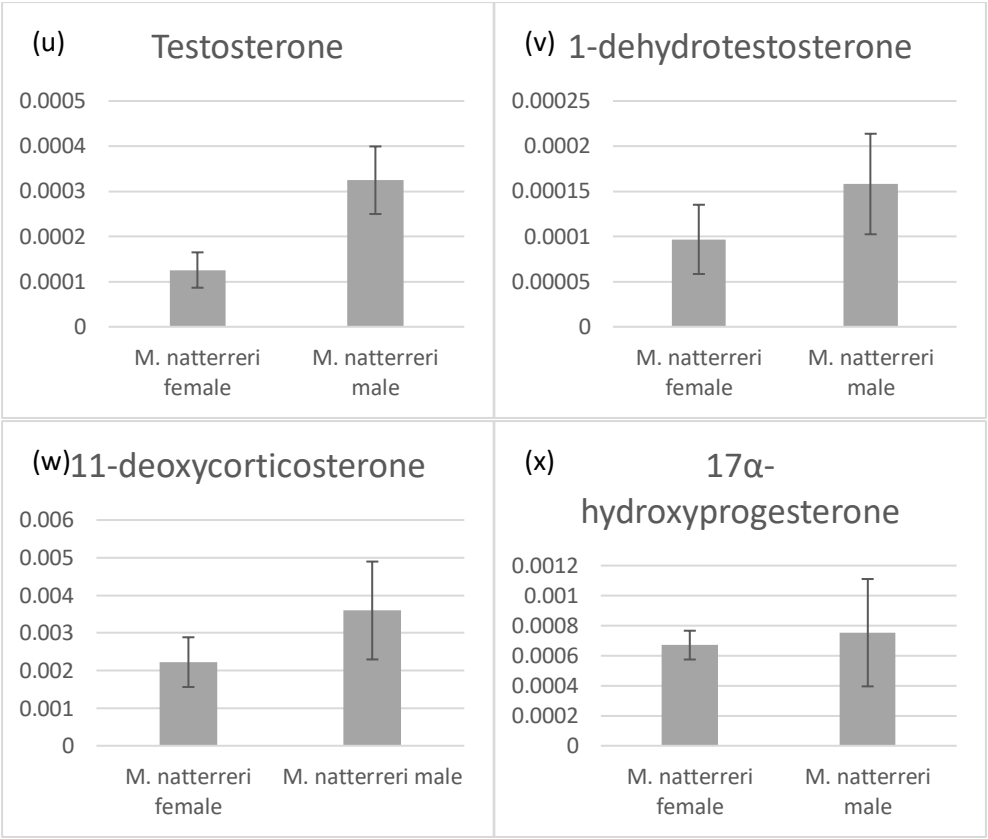

(i) Testosterone
0.006
0.005
0.004
0.003
0.002
0.001
0
M. daubentonii female
M. daubentonii male


(j) 1-dehydrotestosterone
0.0004
0.0003
0.0002
0.0001
0
M. daubentonii female
M. daubentonii male

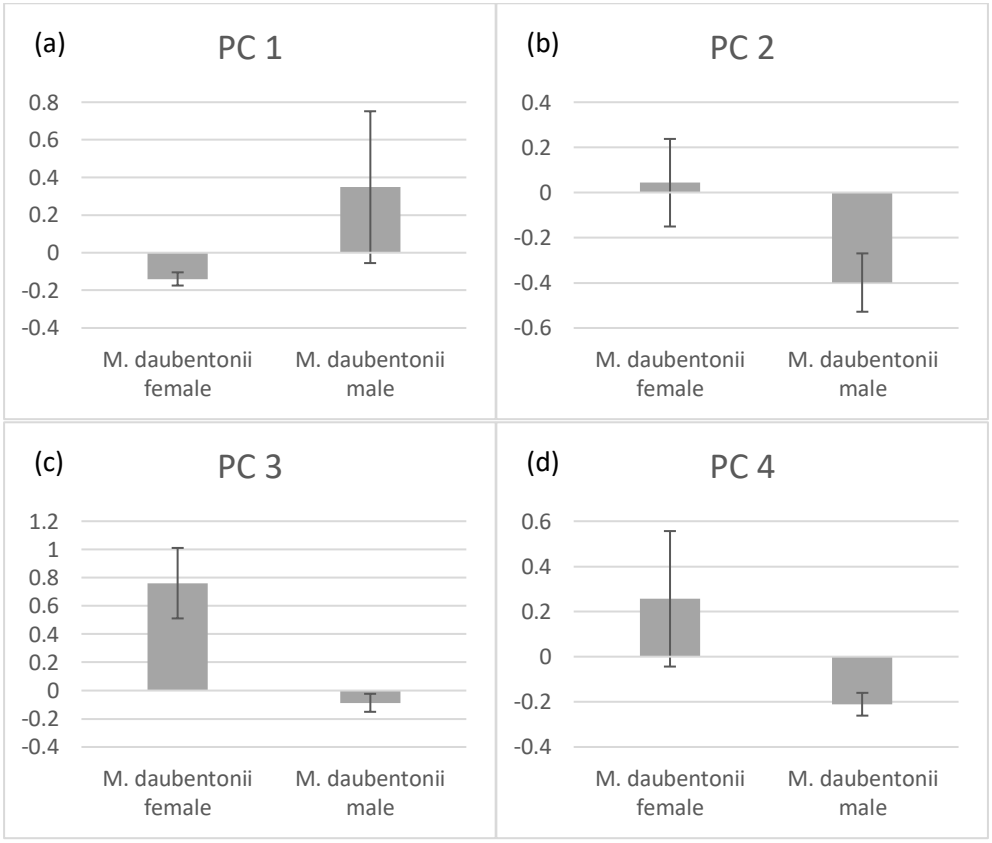

(k) 11-deoxycorticosterone
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
M. daubentonii female
M. daubentonii male

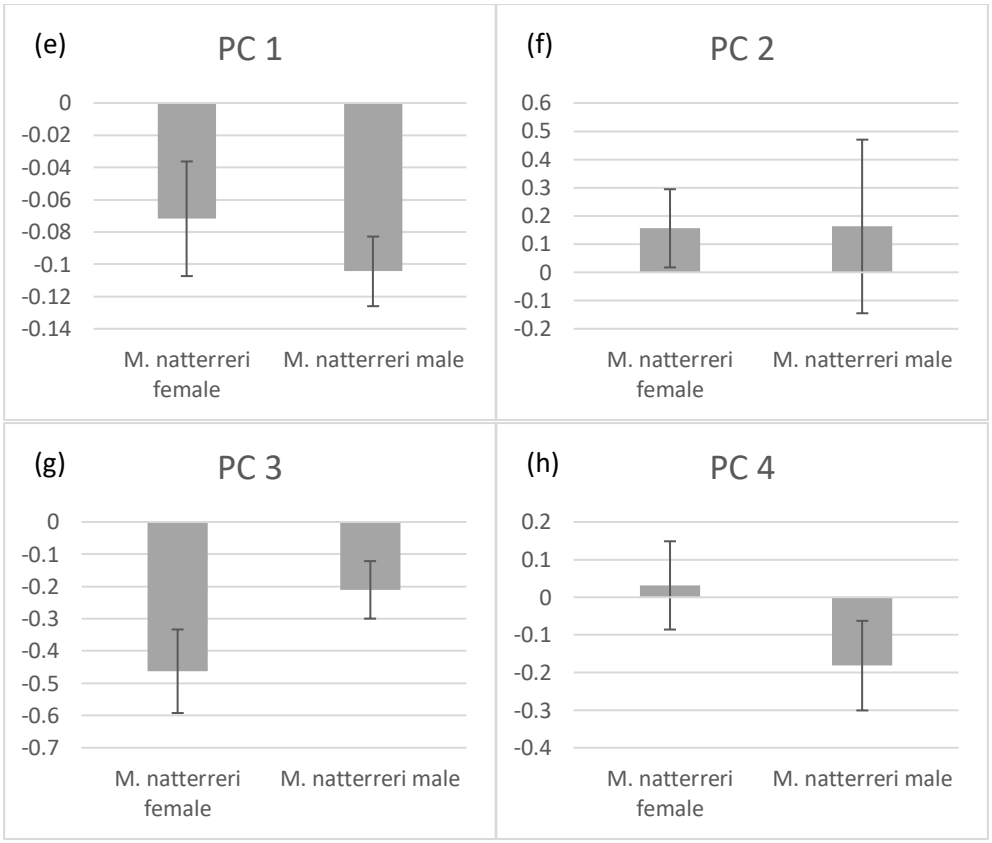

(l) 17α-hydroxyprogesterone
0.04
0.03
0.02
0.01
0
M. daubentonii female
M. daubentonii male


(m)
Aldosterone
0.02
0.015
0.01
0.005
0
M. natterreri female
M. natterreri male
(n)
Androsterone
0.002
0.0015
0.001
0.0005
0
M. natterreri female
M. natterreri male
(o)
Corticosterone
0.0005
0.0004
0.0003
0.0002
0.0001
0
M. natterreri female
M. natterreri male
(p)
Cortisone
0.00012
0.0001
0.00008
0.00006
0.00004
0.00002
0
M. natterreri female
M. natterreri male
(q)
Dihydrotestosterone
0.008
0.006
0.004
0.002
0
M. natterreri female
M. natterreri male
(r)
Hydrocortisone
0.00014
0.00012
0.0001
0.00008
0.00006
0.00004
0.00002
0
M. natterreri female
M. natterreri male
(s)
Pregnenalone
0.03
0.025
0.02
0.015
0.01
0.005
0
M. natterreri female
M. natterreri male
(t)
Progesterone
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0
M. natterreri female
M. natterreri male


(u)
Testosterone
0.0005
0.0004
0.0003
0.0002
0.0001
0
M. natterreri female
M. natterreri male
(v)
1-dehydrotestosterone
0.00025
0.0002
0.00015
0.0001
0.00005
0
M. natterreri female
M. natterreri male
(w)
11-deoxycorticosterone
0.006
0.005
0.004
0.003
0.002
0.001
0
M. natterreri female
M. natterreri male
(x)
17α-hydroxyprogesterone
0.0012
0.001
0.0008
0.0006
0.0004
0.0002
0
M. natterreri female
M. natterreri male

Appendix 4.46 Comparisons between males and females for each principle component; *M. daubentonii* PC1 (a), PC 2 (b), PC 3 (c), PC 4 (d); *M. nattereri* PC1 (e), PC 2 (f), PC 3 (g), PC 4 (h).




(e)
PC 1
0
-0.02
-0.04
-0.06
-0.08
-0.1
-0.12
-0.14
M. natterreri female
M. natterreri male
(f)
PC 2
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
M. natterreri female
M. natterreri male
(g)
PC 3
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
M. natterreri female
M. natterreri male
(h)
PC 4
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
M. natterreri female
M. natterreri male

Acknowledgements

First of all, I'd like to thank James Cresswell. I am deeply grateful for your mentorship; I am a better scientist for it and I could not have completed my PhD without your guidance and support.

One of the best things about research is the opportunity to collaborate with enthusiastic and knowledgeable people. I would like to thank the following people who offered their extensive knowledge and skills. Thank you to Debbie Salmon for helping to develop the protocol for the endocrinological work and guiding me through the laboratory work; Dani Linton and Sadie for our adventures in Wytham Woods; Vicky Fowler for her assistance with fieldwork and Franky Finn for his advice and training in ArcGIS.

There are many friends and colleagues who somehow managed to make the PhD experience not only bearable but fun; Jenn Livesey, Jessica Mulvey, Joanna Kohyt, Julie Day, Luca Mazzini, Paul Craze and Suzanne Richardson; thank you all for your friendship and talking science over coffee. Thank you also to Hugh Warwick and Zoe Broughton, and Esme Taylor, for welcoming me into their homes whilst I was away from mine during fieldwork.

I'd like to thank my funders, The Natural Environment Research Council (NERC), for not only providing the funding for my project but for the many training opportunities they provide.

Many thanks to the Bat Conservation Trust for permission to use data from the National Bat Monitoring Programme. The National Bat Monitoring Programme is run by the Bat Conservation Trust, in partnership with the Joint Nature Conservation Committee, and supported and steered by Natural England, Natural Resources Wales, Northern Ireland Environment Agency, and Scottish Natural Heritage. The NBMP is indebted to all volunteers who contribute data to the programme.

Finally, I'd like to thank my family for their support; Mervyn Travers, Jack Bowhay, Katie Fensome and Sophie Fensome, thank you all for putting up with me. Last but not least, I'd like to thank my mother, Mary E. Bowhay. Thank you for your support

throughout my degree, masters and PhD. I inherited your curiosity and stubbornness, and without those traits, I'd never have started or finished this thesis.